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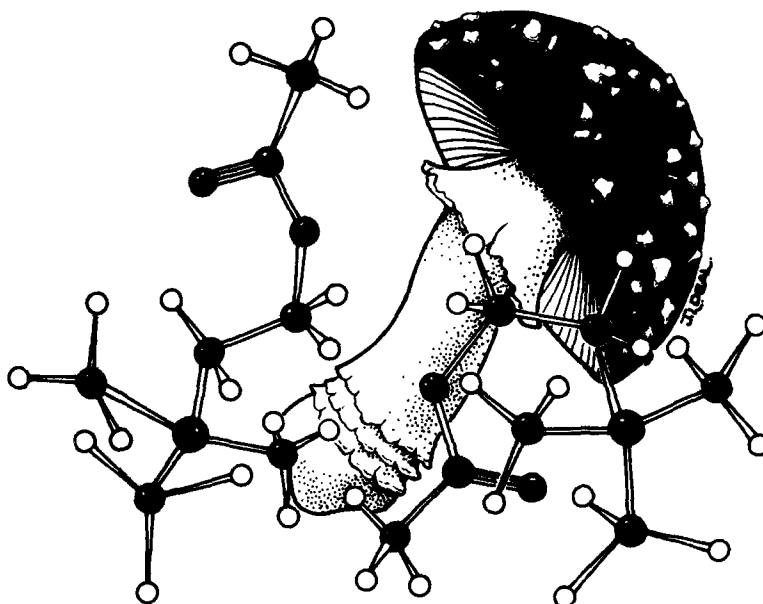
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# Subtypes of Muscarinic Receptors II

Proceedings of the  
Second International Symposium

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Sponsored by  
Boston University School of Medicine  
22-24 August 1985  
Boston, Massachusetts, USA

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Editors  
Ruth R. Levine, Nigel J. M. Birdsall,  
Antonio Giachetti, Rudolf Hammer,  
Leslie L. Iversen, Donald J. Jenden and  
R. Alan North

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<p>Sixteen papers of 20 minute duration were presented during the 2-day period of the Symposium. Fifteen of these papers and the abstracts of all posters have been published in the Proceedings (attached) which appeared as a supplement to the February, 1986 issue of Trends in Pharmacological Sciences (TIPS). Sixty-one names are associated with the authorship of the papers and 135 with that of the poster abstracts.</p> <p>A total of 219 invited scientists registered and attended the symposium (names and addresses attached). The attendance at each of the sessions was excellent and appeared to include all registrants. The 10 minute discussion period between papers was lively, provocative and informative. The authors of posters manned their posters for the entire 4 hour period because there was so much enthusiasm for the work being presented.</p>			
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Block 17 (continued):

Field	Group
06	16

Block 19 (continued)

The Proceedings of the Symposium was sent to about 1,000 scientists a number which included all subscribers to TIPS and all active participants in the Symposium.

Grant support from the U.S. Army Medical Research and Development Command has been acknowledged in the program (copies attached) distributed to all registrants and in the published Proceedings.

The comments of participants at the Symposium and those received in response to the Proceedings indicate great interest in the program in general and in the specific topics and papers of the Symposium. It appears quite evident that the Second Symposium on Subtypes of Muscarinic Receptors has stimulated and is stimulating additional research which should, indeed, lead to the development of new and better therapeutic agents as well as agents useful to the U.S. Army Research and Development Command. The poster papers presented by Army research personnel generated considerable interest and were very well attended.

As another mark of the success of the Second Symposium, the attendees requested a Third Symposium to be held in two years. Planning for another symposium was, therefore, initiated at the meeting and a tentative program is now in place. The Third Symposium will be held in Sydney Australia August 29-31 as a satellite meeting immediately following the Xth International Congress of Pharmacology. Dr. Bebyn Jarrott who attended the Pharmacology Society meetings in Boston and our Second Symposium was so enthusiastic about our Symposium that he recommended a symposium on muscarinic subtypes also be part of the Congress but cover more general topics than the proposed satellite. This has been adopted by the Congress Program Committee.

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Second International Symposium on Subtypes of Muscarinic Receptors

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List of Participants

Registrants, cont.

Dan Capon, Ph.D.  
Molecular Biology  
Genentech, Inc.  
460 Pt. San Bruno Blvd.  
San Francisco, CA 94080

Fulton Crews, Ph.D.  
Department of Pharmacology  
University of Florida  
School of Medicine  
Box J-267 JHMC  
Gainesville, FL 32605

Rose Dagirmanjian, Ph.D.  
Department of Pharmacology & Toxicology  
University of Louisville  
Louisville, KY 40292

Adriaan de Jonge, Ph.D.  
Department of Pharmacology  
Thomae GmbH  
Birkendorferstrabe  
Biberach, 9930  
WEST GERMANY

W.U. Dompert  
Neurobiology Department  
Tropo-Bayer  
Neurather Ring 1  
D 5000 Cologne 80  
WEST GERMANY

R. Ian Fryen, Ph.D.  
Department of Chemistry  
Rutgers University  
Newark, NJ 07012

Bevyn Jarrott, Ph.D.  
Clinical Pharmacology Unit  
University of Melbourne  
Austin Hospital  
Heidelberg 3084  
AUSTRALIA

James J. Kelrns, Ph.D.  
Department of Biochemistry  
Boehringer Ingelheim Pharmaceuticals, Inc.  
90 East Ridge, P.O. Box 368  
Ridgefield, CT 06877

P. Krosgaard-Larsen  
Department of Chemistry  
Royal Danish School of Pharmacy  
2 Universitets Parken  
Copen Hagen DK-2200  
DENMARK

Pedro A. Lehmann, Ph.D.  
Department of Pharmacology & Toxicology  
Center for Research & Advance Studies  
National Polytech Institute  
A.P. 14-740 Mexico 14 0700  
MEXICO

Robert A. Markowitz, Ph.D.  
Department of Psychiatry  
Uniformed Service University  
Jones Bridge Road  
Bethesda, MD 20854

M. Matlib, Ph.D.  
Department of Pharmacology  
and Cell Biophysics  
University of Cincinnati  
231 Bethesda  
Cincinnati, OH 45241

Mary Harris Rice, Ph.D.  
38 Spring Circle  
Shrewsbury, MA 01545

Atko Sawa, Ph.D.  
Department of Pharmacology  
University of Texas  
7428 Louis Pasteur Drive #1608  
San Antonio, TX 78229

Daniel Smer, M.D.  
Corporate Medical Department  
Zamban Group  
Brasso  
ITALY

Robert W. Sikes, Ph.D.  
Department of Anatomy  
Boston University  
School of Medicine  
80 East Concord Street  
Boston, MA 02118

Registrants, cont.

Erick T. Suen, Ph.D.  
Department of Pharmacology  
Nova Pharmaceuticals Corporation  
5210 Eastern Avenue  
Baltimore, MD 21224

Amin Suria, Ph.D.  
Department of Pharmacology  
AGA Khan University  
Stadium Road, P.O. Box 3500  
Karachi  
PAKISTAN

William Wardell, Ph.D.  
Department of Medicine  
Boehringer Ingelheim Pharmaceuticals, Inc.  
90 East Ridge Road, P.O. Box 368  
Ridgefield, CT 06877

Karl Heniz Weber, Ph.D.  
Department of Medical Chemistry  
Boehringer Ingelheim KG  
Ingelheim  
WEST GERMANY

Carmille Wermuth, Ph.D.  
University of Strasbourg  
Centre de Neurosciences  
5 rue Blaise Pascal  
Strasbourg 6700  
FRANCE

Virginia Zaratzian, Ph.D.  
USDA/FSIS  
300 12th Street S.W.  
Washington, D.C. 20250

Registrants, cont.

B. V. Rama Sastry, D.Sc., Ph.D.  
Department of Pharmacology  
Vanderbilt University  
Medical Center, North  
Nashville, TN 37232

John Saunders, Ph.D.  
Department of Medicinal Chemistry  
Merck, Sharp and Dohme  
Neuroscience Research Centre  
Terlings Park, Eastwick Road  
Harlow, Essex  
ENGLAND

Carmelo Scarpignato, M.D.  
Department of Pharmacology  
University of Parma  
Univpr 530327  
ITALY

Joel B. Schachter, Ph.D.  
Department of Pharmacology  
University of Pennsylvania  
Hamilton Walk G-3  
Philadelphia, PA

Michael Schimerlik, M.D.  
Department of Biochemistry and Biophysics  
Oregon State University  
Corvallis, OR 97331-6503

Miriam Schweber, Ph.D.  
Department of Biochemistry  
Boston University School of Medicine  
80 E. Concord Street  
Boston, MA 02118

Jerry Sepinwall, Ph.D.  
Department of Pharmacology  
Hoffmann-La Roche Inc.  
Bldg. 76, Rm 627  
Nutley, NJ 07110

Vimala H. Sethy, Ph.D.  
CNS Unit  
The Upjohn Company  
7000 Portage Road  
Kalamazoo, MI 49001

Nigel Paul Shankley, Ph.D.  
Dept. of Analytical Pharmacology  
Rayne Institute, King's College  
School of Medicine & Dentistry  
123, Coldharbour Lane  
London, SE5 9NU  
UNITED KINGDOM

Gregory M. Shutske, Ph.D.  
Dept. of Chemical Research  
Hoechst-Roussel Pharmaceuticals Inc.  
Route 202-206 North  
Somerville, NJ 08876

Robert W. Sikes, Ph.D.  
Department of Anatomy  
Boston University School of Medicine  
80 E. Concord Street  
Boston, MA 02118

Stephen Sims, Ph.D.  
Department of Physiology  
University of Massachusetts  
Medical School  
55 Lake Avenue, North  
Worcester, MA 01605

F. Marott Sinex, Ph.D.  
Department of Biochemistry  
Boston University School of Medicine  
80 East Concord Street  
Boston, MA 02118

Joshua J. Singer, M.D.  
Dept. of Physiology  
University of Massachusetts  
Medical School  
55 Lake Avenue, North  
Worcester, MA 01605

Emil R. Smith, Ph.D.  
Dept. of Pharmacology  
University of Massachusetts  
Medical School  
55 Lake Avenue, North  
Worcester, MA 01605

Registrants, cont.

Charles J. Paget, Ph.D.  
Department of Chemical Research  
Eli Lilly and Company  
Lilly Corporate Center  
Indianapolis, IN 46285

Achilles J. Pappano, Ph.D.  
Department of Pharmacology  
University of Connecticut Health Center  
263 Farmington Avenue  
Farmington, CT 06032

Charles C. Pendley, Ph.D.  
Department of Pharmacology  
W. H. Rorer  
500 Virginia Drive  
Ft. Washington, PA 19034

Judith Maria B. Pinto  
Department of Pharmacology  
Massachusetts College of Pharmacy  
179 Longwood Avenue  
Boston, MA 02115

Lincoln T. Potter, M.D.  
Department of Pharmacology  
University of Miami  
P.O. Box 016189  
Miami, FL 33101

Reni Quirion, M.D.  
Douglas Hospital Research Centre  
McGill University  
6875 Blvd Lasalle  
Verdun, Quebec  
CANADA H4H 2R3

Sten Ramsby, Ph.D.  
Research & Development Laboratories  
Astra Lakemedel AB  
15185 Sodertalje  
SWEDEN

Elliott Richelson, M.D.  
Department of Psychiatry and Pharmacology  
Mayo Clinic and Foundation  
200 First Street S.W.  
Rochester, MN 55905

Donald Kay Riker, Ph.D.  
Department of Exploratory Research  
Richardson-Vicks, Inc.  
1 Far Mill Crossing  
Shelton, CT 06484

Bjorn Ringdahl, Ph.D.  
Department of Pharmacology  
University of California/L.A.  
School of Medicine  
Center for the Health Sciences  
Los Angeles, CA 90024

F.F. Roberts, Ph.D.  
Department of Neuropharmacology  
Glaxo Group Research Ltd.  
Priory Street  
Ware Hertfordshire, SG12 0DJ  
UNITED KINGDOM

William R. Roeske, M.D.  
Internal Medicine and Pharmacology  
University of Arizona  
1501 N. Campbell Avenue  
Tucson, AZ 85724

Gary C. Rosenfeld, Ph.D.  
Department of Pharmacology  
University of Texas  
Medical School  
P.O. Box 20708  
Houston, TX 77025

Karen G. Rothberg, Ph.D.  
John B. Pierce Foundation  
290 Congress Avenue  
New Haven, CT 06519

Jean-Francois Ruisigny, M.D.  
Centre De Recherche Delalande  
10, Rue des Carrieres  
Rueil-Malmaison 92500  
FRANCE

Antonio Sastre, Ph.D.  
Department of Physiology & Neuroscience  
John Hopkins School of Medicine  
725 N. Wolfe Street  
Baltimore, MD 21203

Registrants, cont.

Suzanne B. McMaster, Ph.D.  
Department of Neurotoxicology  
USAMRICD  
SGRD-UV-YN/McMaster  
Aberdeen Proving Ground, MD 21010-5425

William S. Messer, Ph.D.  
Center for Brain Research, Box 605  
University of Rochester  
575 Elmwood Avenue  
Rochester, NY 14642

Bruce Miller, M.D.  
Department of Neurology  
University of California/L.A.  
School of Medicine  
710 Westwood Plaza  
Los Angeles, CA 90024

G. Nabi Mir, Ph.D.  
Department of Pharmacology  
Ayerst Laboratories Research, Inc.  
CN 8000  
Princeton, NJ 08540

Ulrich Mittmann, M.D.  
Department of Pharmacology  
c/o Karl Thomae GmbH  
Birkendorfer Str. 65  
D-7950 Biberach  
WEST GERMANY

Frederick J. Monsma  
Center for Brain Research  
University of Rochester  
School of Medicine and Dentistry  
601 Elmwood Avenue  
Rochester, NY 14642

Walter H. Moos, Ph.D.  
Department of Medicinal Chemistry  
Warner-Lambert/Parke-Davis  
2800 Plymouth Road  
Ann Arbor, MI 48105

Mary A. Nastuk, Ph.D.  
Dept. of Psychology & Brain Sciences  
Massachusetts Institute of Technology  
E25-618  
Cambridge, MA 02139

Neil M. Nathanson, Ph.D.  
Department of Pharmacology, SJ-30  
University of Washington  
Seattle, WA 98195

Kenneth J. Nichol, Ph.D.  
Dept. of Pharmaceutical Chemistry  
The Boots Company Plc  
Pennyfoot Street, R4  
Nottingham NG2 3AA  
UNITED KINGDOM

Lalita Noronha-Blob, Ph.D.  
Dept. of Molecular Pharmacology  
Nova Pharmaceutical Corp.  
5210 Eastern Avenue  
Baltimore, MD 21224

R. Alan North, M.D., Ph.D.  
Nutrition & Food Science  
77 Massachusetts Avenue  
Cambridge, MA 02139

Gary L. Olson, Ph.D.  
Medicinal Chemistry Department II  
Hoffmann-La Roche Inc.  
340 Kingsland Street  
Nutley, NJ 07110

Richard W. Olsen, Ph.D.  
Department of Pharmacology  
UCLA School of Medicine  
Los Angeles, CA 90024

J. Mark Ordy, Ph.D.  
Pennwalt Corporation  
755 Jefferson Road  
Rochester, NY 14623

Chug Owqyng, M.D.  
Department of Internal Medicine  
University of Michigan  
Medical School  
Ann Arbor, MI 48109

Ferdinando Pagani, Ph.D.  
Department of Pharmacology  
Istituto de Angeli S.p.A.  
Via Serio 15  
20139 Milano  
ITALY

Registrants, cont.

Dexter S. Louie, Ph.D.  
Department of Internal Medicine  
University of Michigan  
R6687 Kresge 1  
Ann Arbor, MI 48103

Gary R. Luthin, Ph.D.  
Department of Pharmacology  
University of Pennsylvania  
School of Medicine  
Philadelphia, PA 19104

Hans Machleidt, Ph.D.  
Dr. Karl Thomae GmbH  
Birkendorfer StraBe 65  
D-7950 Biberach 1  
WEST GERMANY

Frank C. MacIntosh, Ph.D.  
Department of Physiology  
McGill University  
3655 Drummond Street  
Montreal Quebec  
CANADA H3G 1Y6

James E. Mack, Ph.D.  
Department of Pharmacology  
University of Mississippi  
School of Pharmacy  
University, MS 38677

Richard M. Mangano, Ph.D.  
Department of Pharmacology  
Boffmann-La Roche Inc.  
Kingsland Street  
Nutley, NJ 07110

Richard A. Margolin, Ph.D.  
Department of Psychiatry  
Vanderbilt University  
A-2215 Medical Center North  
Nashville, TN 37232

Michael Marks, Ph.D.  
Institute for Behavioral Genetics  
University of Colorado  
Campus Box 447  
Boulder, CO 80309

Deborah C. Mash, Ph.D.  
Department of Neurology  
Beth Israel Hospital  
330 Brookline Avenue  
Boston, MA 02215

Donald R. Maxwell, Ph.D.  
Preclinical Research  
Warner-Lambert Company  
2800 Plymouth Road  
Ann Arbor, MI 48105

John Marvin May, M.D.  
Department of Pharmacology  
Emory University  
School of Medicine  
Clifton Rd.  
Atlanta, GA 30322

Narbert Mayer, Ph.D.  
Department of Biochemistry  
Dr. Karl Thomae GmbH  
Birkemolorter-Strane  
Biberach 7950  
WEST GERMANY

Karen McComack, Ph.D.  
Department of Pharmacology  
Boechst-Roussel Pharmaceuticals  
Rt 202-206 North  
Somerville, NJ 08876

David A. McCormick, Ph.D.  
Department of Neurology, Room C338  
Stanford University Medical Center  
Stanford, CA 94305

Michael McKinney, Ph.D.  
Department of Pharmacology  
Mayo Foundation  
200 First Street, SW  
Rochester, MN 55905

Kathryn K. McMahon, Ph.D.  
Department of Pharmacology  
University of South Carolina  
School of Medicine  
Columbia, SC 29208

Registrants, cont.

Marilyn Halonen, Ph.D.  
Division of Respiratory Sciences  
University of Arizona  
Health Science Center  
Tucson, AZ 85724

Harriet W. Hamilton, Ph.D.  
Chemistry Department  
Warner Lambert/Parke Davis  
2800 Plymouth Road  
Ann Arbor, MI 48118

Rudolf Hammer, Ph.D.  
Boehringer Ingelheim Zentrale GmbH  
6507 Ingelheim am Rhein  
WEST GERMANY

T. Kendall Harden, Ph.D.  
Department of Pharmacology  
University of North Carolina at Chapel Hill  
Faculty Laboratory Office, Building 231B  
Chapel Hill, NC 27514

Basil I. Hirschowitz, M.D.  
Department of Gastroenterology  
University of Alabama  
Birmingham, AL 35294

Wayne P. Hoss, Ph.D.  
Center for Brain Research  
University of Rochester Medical Center  
601 Elmwood Avenue  
Rochester, NY 14642

Ervin Horvath, Ph.D.  
Neurobiology Department  
Tropenwerke  
Neurather Ring 1  
D-5000 Cologne  
WEST GERMANY

E.P. Huger, Ph.D.  
Department of Biochemistry  
Roche-Roussel Pharmaceuticals  
Rt. 202-206 N  
Somerville, NJ 08876

Stephen Burt, Ph.D.  
DuPont Nem-Products  
549 Albany Street  
Boston, MA 02118

Dale Hunter  
Department of Pharmacology  
University of Washington  
SJ-30  
Seattle, WA 98195

Deborah K. Hyslop, Ph.D.  
Pre-clinical Central Nervous System Research  
Bristol-Myers Co.  
2404 Pennsylvania Avenue  
Evansville, IN 47721

Leslie Iversen, Ph.D.  
Merck, Sharp & Dohme  
Neuroscience Research Center  
Terlings Park, Eastwick Road  
Harlow, Essex CM 20 QR  
UNITED KINGDOM

Donald J. Jenden, Ph.D.  
Department of Pharmacology  
University of California/L.A.  
School of Medicine  
Los Angeles, CA 90024

J.A. Joseph, Ph.D.  
Department of Behavioral Sciences  
Armed Forces Radiobiology Res. Inst.  
Bethesda, MD 20814

Jean-Paul Kan, M.D.  
Department of Neurobiology  
Centre de Recherches CLIN-MIDY  
Rue du Pr. Joseph Blayac  
Montpellier 34082  
FRANCE

Harvey R. Kaplan, Ph.D.  
Department of Pharmacology  
Warner-Lambert/Parke-Davis  
2800 Plymouth Road  
Ann Arbor, MI 48105

Paul V. Kaplita, Ph.D.  
Department of Physiological Pharmacology  
Nova Pharmaceutical Corporation  
5210 Eastern Avenue  
Baltimore, MD 21224-2788

Registrants, cont.

Manfred Karobath, M.D.  
Preclinical Research  
Sandoz, Ltd., Bldg. 386/216  
Basel CH-4002  
SWITZERLAND

Ester D. Katz, M.D.  
Department of Pharmacology  
University of California  
School of Medicine  
Los Angeles, CA 90024

Eva King Killam, Ph.D.  
Department of Pharmacology  
University of California  
School of Medicine, Med Sci 1  
Davis, CA 95616

Keith F. Killam, Ph.D.  
Department of Pharmacology  
University of California  
School of Medicine, Med Sci 1  
Davis, CA 95616

William Kinnier, Ph.D.  
Nova Pharmaceutical Corporation  
5210 Eastern Avenue  
Baltimore, MD 21224

Haruo Kobayashi, Ph.D.  
Department of Physiology  
Tokyo Medical College  
1-1, Shinjuku-6-Chome,  
Shinjuku-ku  
Tokyo 160  
JAPAN

Herbert Ladinsky, Ph.D.  
Department of Biochemistry  
Istituto de Angeli S.p.A.  
Via Serio 15  
Milano 20139  
ITALY

Kristine A. Erickson-Lamy, M.D.  
Howe Laboratory of Ophthalmology  
Harvard Medical School  
243 Charles Street  
Boston, MA 02114

Alan S. Lane, M.D.  
Hopen and Lane, M.D.'s P.A.  
3419 Johnson Street  
Hollywood, FL 33021

John D. Lane, Ph.D.  
Department of Pharmacology  
Texas College of Osteopathic Medicine  
Camp Bowie at Montgomery  
Ft. Worth, TX 76107

Michel Langlois, M.D.  
Centre De Recherche Delalande  
10, rue des Carrieres  
Rueil Balmaison 92500  
FRANCE

H. J. Leighton, Ph.D.  
Department of Pharmacology  
Burroughs Wellcome Co.  
3030 Cornwallis Road  
Research Triangle Park, NC 27709

Ruth R. Levine, Ph.D.  
Division of Medical & Dental Sciences  
Boston University School of Medicine  
80 E. Concord Street  
Boston, MA 02118

Leonard Lichtblau, Ph.D.  
Department of Pharmacology  
University of Minnesota  
435 Delaware Street, SE  
Minneapolis, MN 55455

Arnold S. Lippe, Ph.D.  
Matrix Research Labs  
N. Academic Complex, Rm. 7/232  
City College of New York  
New York, NY 10031

Walter Londong, M.D.  
Chirurg. Klinik Innenstadt and  
Chirurg. Poliklinik  
University of Munich  
NussbaumstraBe 20  
D-8000 Munich  
WEST GERMANY

Registrants, cont.

Stephen Fisher, Ph.D.  
Neuroscience Laboratory  
University of Michigan  
1103 E. Huron  
Ann Arbor, MI 48109

Donna D. Flynn, Ph.D.  
Department of Pharmacology  
University of Miami  
School of Medicine  
P.O. Box 016189  
Miami, FL 33101

Jo-Ann E. T. Fox, M.D.  
School of Nursing  
McMaster University  
1200 Main Street West  
Hamilton, Ontario  
CANADA L8N 3Z5

Paul H. Franklin, Ph.D.  
Center for Brain Research  
601 Elmwood Avenue  
Rochester, NY 14642

Stephen B. Freedman, Ph.D.  
Department of Biochemistry  
Merck, Sharp and Dohme Research Labs.  
Terlings Park, Eastwick Road  
Harlow, Essex CM20 2QR  
UNITED KINGDOM

Hermann Fuder, M.D.  
Department of Pharmacology  
University of Mainz  
Obere Zahlbacher Str. 67  
D-6500 Mainz  
WEST GERMANY

Marcello Gaetani, M.D.  
R & D Director  
Istituto de Angeli S.p.A.  
Via Serio 15  
20139 Milan  
ITALY

Antonio Giachetti, Ph.D.  
Istituto de Angeli S.p.A.  
Via Serio 15  
20139 Milano  
ITALY

Ettore Giraldo, Ph.D.  
Department of Biochemistry  
Istituto de Angeli S.p.A.  
Via Serio 15  
Milano 20139  
ITALY

Per Gjorstrup, M.D.  
Department of Pharmacology  
AB Hassle  
Karragatanb  
Molndal S-43183  
SWEDEN

Barry I. Gold, Ph.D.  
Anaquest  
100 Mountain Avenue  
Murry Hill, NJ 07974

Rueben Gonzales, Ph.D.  
Department of Pharmacology  
University of Florida  
Box J-267, JHMHC  
Gainesville, FL 32610

Richard K. Gordon, Ph.D.  
Department of Applied Biochemistry  
Walter Reed Army Institute of Research  
Building 40, Room 1034  
Washington, D.C. 20307-5100

Sharon R. Grady, M.D.  
Institute for Behavioral Genetics  
University of Colorado  
Campus Box 447  
Boulder, CO 80309

Ann M. Graybiel  
Department of Psychology  
Massachusetts Institute of Technology  
E25-618  
Cambridge, MA 02139

Ronald C. Griffith, Ph.D.  
Pennwalt Corporation  
Pharmaceutical Division  
755 Jefferson Road  
Rochester, NY 14603

Registrants, cont.

James Douglas, Ph.D.  
John B. Pierce Foundation  
290 Congress Avenue  
New Haven, CT 06519

Barry Dubinsky, Ph.D.  
Department of Biological Research  
Ortho Pharmaceutical Corp.  
P.O. Box 300  
Raritan, NJ 08869-0602

Wolfgang Eberlein, Ph.D.  
c/o Dr. Karl Thomae GmbH  
Postfach 1755  
Birkendorfer StraBe 65  
D-7950 Biberach 1  
WEST GERMANY

Terrance Egan  
Dept. of Applied Biological Sciences  
Massachusetts Institute of Technology  
Cambridge, MA 02139

Richard M. Eglen, Ph.D.  
Department of Pharmacology  
Syntex Research Centre  
Beriot-Watt University  
Riccarton  
Edinburgh EH14 4AS  
UNITED KINGDOM

Frederick J. Ehlert, Ph.D.  
Department of Pharmacology  
University of California/L.A.  
School of Medicine  
Los Angeles, CA 90024

Bengt Ek, M.D.  
Dept. of Pharmacology & Biochemistry  
AB Hassle  
S-43183 Molndal  
SWEDEN

John T. Elder, Ph.D.  
Department of Pharmacology  
Creighton University  
2500 California Street  
Omaha, NE 68178

John Ellis, Ph.D.  
Department of Psychiatry  
University of Vermont  
Burlington, VT 05405

Masao Endoh, M.D.  
Department of Pharmacology  
Tohoku University  
School of Medicine  
2-1 Seiryomachi  
Sendai 980  
JAPAN

Wolfhard Engel, M.D.  
c/o Dr. Karl Thomae GmbH  
Postfach 1755  
Birkendorfer StraBe 65  
D-7950 Biberach 1  
WEST GERMANY

K. Ensing, M.D.  
Department of Toxicology  
A. Deusinglaan 2  
AW Groningen 9713  
NETHERLANDS

Belmut A. Ensinger, Ph.D.  
Department of Biochemistry  
Boehringer Ingelheim KG  
Binger Str. 173  
6507 Ingelheim  
WEST GERMANY

Stuart Fielding, Ph.D.  
Department of Pharmacology  
Hoechst-Roussel Pharmaceuticals, Inc.  
Rt. 202-206 North  
Somerville, NJ 08876

Margaret G. Filbert, Ph.D.  
Department of Neurotoxicology  
USAMRICD  
SGRD-UV-YN  
Aberdeen Proving Ground, MD 21010

Joan Fiore, Ph.D.  
Department of Pharmacology  
Hoechst-Roussel Pharmaceuticals, Inc.  
Rt. 202-206 North  
Somerville, NJ 08876

Registrants, cont.

David A. Brown, Ph.D.  
University of London  
School of Pharmacy  
29/39 Brunswick Square  
London, WC1N 1AX  
UNITED KINGDOM

Joan Heller Brown, Ph.D.  
Department of Medicine/Pharmacology  
Univ. of California/San Diego, M-013-H  
La Jolla, CA 92093

Oliver M. Brown, Ph.D.  
Department of Pharmacology  
SUNY Upstate Med. Cent.  
766 Irving Avenue  
Syracuse, NY 13210

Noel J. Buckley, Ph.D.  
Laboratory of Cell Biology  
National Institute of Mental Health  
Parklawn Building, 5600 Fishers Lane  
Bethesda, MD 20205

Sir Arnold Burgen, M.D.  
Darwin College  
Cambridge University  
Cambridge, CB3 9EU  
UNITED KINGDOM

Henry F. Campbell, M.D.  
Medicinal Chemistry Department  
William B. Rorer, Inc.  
500 Virginia Drive  
Fort Washington, PA 19034

John M. Carney, Ph.D.  
Department of Pharmacology  
University of Oklahoma  
Health Science Center  
P.O. Box 26901  
Oklahoma City, OK 73190

Gerald O. Carrier, Ph.D.  
Department of Pharmacology & Toxicology  
Medical College of Georgia  
1459 Laney-Walker Boulevard  
Augusta, GA 30912

Richard E. Chipkin, Ph.D.  
Department of Pharmacology  
Schering Corporation  
60 Orange Street  
Bloomfield, NJ 07003

Stephen M. Collins, M.D.  
Department of Medicine  
McMaster University Medical Centre  
1200 Main Street West  
Hamilton, Ontario  
CANADA L8N 3Z5

Linda L. Coughenour, Ph.D.  
Department of Pharmacology  
Warner-Lambert/Parke Davis  
2800 Plymouth Road  
Ann Arbor, MI 48105

Joseph Coupet, Ph.D.  
Lederle Laboratories Med. Res.  
American Cyanamid  
North Middletown  
Pearl River, NY 10965

Ernest A. Daigneault, Ph.D.  
Department of Pharmacology  
East Tennessee State University  
Quillen-Dishner College of Medicine  
P.O. Box 19810A  
Johnson City, TN 37614

E.E. Daniel, M.D.  
McMaster University  
Faculty of Health Sciences  
Department of Neurosciences  
1200 Main Street West  
Hamilton, Ontario  
CANADA L8N 3Z5

Joan DeCosimo, Ph.D.  
Department of Pharmacology  
Boehringer-Ingelheim Pharmaceuticals  
Rt. 202-206 N.  
Somerville, NJ 08876

Edward F. Domino, M.D.  
Department of Pharmacology  
University of Michigan  
M5414 Medical Science, Bldg. I  
Ann Arbor, MI 48109

**REGISTRANTS**  
**Second Symposium on Subtypes of Muscarinic Receptors**

Abdu-Adem, M.D.  
Department of Pharmacology  
University of Arizona  
College of Medicine  
1501 N. Campbell Avenue  
Tucson, AZ 85724

Ateeq Ahmad, M.D.  
Department of Biochemistry  
Walter Reed Army Institute of Research  
Building 40  
Washington, D.C. 20307-5100

Pam Albaugh-Robertson, M.D.  
Department Bioorganic Chemistry  
Allergan Pharmaceuticals  
2525 Dupont Drive  
Irvine, CA 92713

R. Amstutz, M.D.  
Preclinic Research  
Sandoz Ltd.  
Basel CH4002  
SWITZERLAND

Betty Anthony, Ph.D.  
Department of Pharmacology & Toxicology  
Medical College of Georgia  
Laney-Walker Blvd.  
Augusta, GA 30912

Linda Antonian, Ph.D.  
Matrix Research Labs  
North Academic Complex, 7/232  
City College of New York  
New York, NY 10031

Robert S. Aronstam, Ph.D.  
Department of Pharmacology & Toxicology  
Medical College of Georgia  
Laney-Walker Blvd.  
Augusta, GA 30912

Hillary Barnett  
Department of Pharmacology  
Northeastern University  
396 Langley Rd. #1  
Newton Centre, MA 02159

Steven I. Baskin, Ph.D.  
Department of Physiology  
USAMRICD  
SGRD-UV-YP  
Aberdeen Proving Ground, MD 21010-5425

S. Batra, M.D.  
Department of Obstetrics & Gynecology  
University Hospital  
Lund S-22185  
SWEDEN

Paul Bianchi, M.D.  
Department of Pharmacology  
Thomas Jefferson University  
1020 Locust Street  
Philadelphia, PA 19107

Nigel Birdsall, Ph.D.  
Division of Molecular Pharmacology  
National Institute for Medical Research  
The Ridgeway, Mill Hill  
London NW7-1AA  
UNITED KINGDOM

Kathleen Biziere, M.D.  
Department of Neurobiology  
Centre de Recherches  
Clin-Midy  
Rue de Pr. Joseph Blayac  
Montpellier 34082  
FRANCE

James C. Blosser, Ph.D.  
Department of Pharmacology  
Pennwalt Corporation  
Pharmaceutical Division  
755 Jefferson Road  
Rochester, NY 14623

Clarence A. Broomfield, Ph.D.  
Department of Basic Pharmacology  
U.S. Army Medical  
Institute of Chemical Defense  
Aberdeen Proving Ground, MD 21010

# Subtypes of Muscarinic Receptors II

Proceedings of the Second International Symposium

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Boston, Massachusetts, USA

## Editors

Ruth R. Levine, Nigel J. M. Birdsall, Antonio Giachetti, Rudolf Hammer,  
Leslie L. Iversen, Donald J. Jenden and R. Alan North

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### **Symposium co-ordinator**

Dr Ruth R. Levine  
Graduate Biomedical Sciences  
Studies  
Boston University School of  
Medicine  
80 East Concord Street  
Boston, MA 02218, USA

### **Scientific committee**

Dr Nigel J. M. Birdsall  
Division of Physical Biochemistry  
National Institute for Medical  
Research  
The Ridgeway, Mill Hill  
London, NW7 1AA, UK

Dr Antonio Giachetti  
Istituto De Angeli S.p.A.  
Via Serio 15  
20139 Milano, Italy

Dr Rudolf Hammer  
Boehringer Ingelheim Zentrale  
GmbH  
6507 Ingelheim am Rhein  
West Germany

Dr Leslie Iversen  
Merck, Sharp & Dohme  
Research Laboratories  
Neuroscience Research Centre  
Terlings Park, Eastwick Road  
Harlow, Essex CM 20 QR, UK

Dr Donald J. Jenden  
Department of Pharmacology  
University of California  
School of Medicine  
Los Angeles, CA 90024, USA

Dr R. Alan North  
Nutrition & Food Science  
Massachusetts Institute of  
Technology  
77 Massachusetts Avenue  
Cambridge, MA 02139, USA

### **Symposium speakers**

Dr David A. Brown  
Department of Pharmacology  
The School of Pharmacy  
University of London  
29/39 Brunswick Square  
London, WC1N 1AX, UK

Dr E. E. Daniel  
Department of Neuroscience  
Faculty of Health Sciences  
McMaster University  
1200 Main Street, West  
Hamilton, Ontario L8N 3Z5  
Canada

Dr Stephen Fisher  
Neuroscience Laboratory  
University of Michigan  
1103 E. Huron  
Ann Arbor, MI 48109, USA

Dr T. Kenda!! Harden  
Department of Pharmacology  
University of North Carolina at  
Chapel Hill  
Faculty Laboratory Office, Building  
231H  
Chapel Hill, NC 27514, USA

Dr Manfred Karobath  
Pharmaceutical Division  
Preclinical Research  
Sandoz Ltd.  
CH-4002 Basle, Switzerland

Dr David A. McCormick  
Department of Neurology, Room  
C338

Stanford University Medical Center  
Stanford, CA 94305, USA

Dr Michael Schimerlik  
Department of Biochemistry and  
Biophysics  
Oregon State University  
Corvallis, OR 97331-6503, USA

at present on sabbatical  
c/o Professor James Dahlberg  
Department of Physiological  
Chemistry  
University of Wisconsin  
553 Bardeen Labs  
Madison, WI 53706, USA

Dr Stephen Sims  
Department of Physiology  
University of Massachusetts  
Medical School  
55 Lake Avenue, North  
Worcester, MA 01065, USA

Dr Mordechai Sokolovsky  
Department of Pharmacology  
Tel Aviv University  
Tel Aviv, Israel

Dr Annmarie Suprenant  
Gastroenterology Division  
Department of Medicine  
Beth Israel Hospital  
Harvard Medical School  
330 Brookline Avenue  
Boston, MA 02215, USA

Dr Henry I. Yamamura  
Department of Pharmacology  
The University of Arizona  
College of Medicine  
Arizona Health Sciences Center  
Tucson, AZ 85724, USA

## ACKNOWLEDGEMENTS

The organizers of the Second Symposium on Subtypes of Muscarinic Receptors acknowledge with appreciation the support of the following for the Symposium and the publication of the proceedings.

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## GLOSSARY OF ABBREVIATIONS

ACh	Acetylcholine	i.p.s.p.	Inhibitory postsynaptic potential
ADP	Adenosine diphosphate	$K_a$	Association constant
AF-DX-116	(11[[2-[(diethylamino) methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido [2,3-b][1,4]benzodiazepine-6-) one	$K_d$ or $K_D$	Dissociation constant
	Afterhyperpolarization	$K_i$	Inhibition constant
a.h.p.	An enzyme inhibitor	L-	Low affinity agonist form of the mAChR for agonists
Antipain	Batrachotoxin	mAChR	Muscarinic acetylcholine receptor
BTX	Choline acetyltransferase	M-current	Membrane potassium current controlled by muscarinic receptor.
CAT or ChAT	Carbachol, a cholinergic agonist		Abbreviated $I_M$ or $I_{K,M}$
CCh	Cis-methyldioxolane. When labeled with tritium ( $[^3H]$ ) (+) CD used for binding studies	McN-A-343	4-( <i>m</i> -Chlorophenylcarbamoyloxy)-2-butylnyltrimethyl ammonium, a muscarinic agonist
CD or (+) CD	Cytidine diphospho-diacylglycerol	<i>N</i> -protein	Protein associated with receptors for various hormones and neurotransmitters which binds GTP
CDP-DAG	Adenosine-3'-5'-cyclic-monophosphate	NEM	<i>N</i> -Ethylmaleimide, a compound which reacts with sulfhydryl groups and other nucleophiles
Cyclic AMP (cAMP)	Guanosine-3'-5'-cyclic-monophosphate	4NMPB	<i>N</i> -methyl-4-piperidyl benzilate
Cyclic GMP (cGMP)	Gallopamil or methoxyverapamil, a calcium antagonist	NMS	<i>N</i> -methylscopolamine, a muscarinic antagonist. When labeled with tritium ( $[^3H]$ -NMS) used for binding studies
D-600	Diacylglycerol		Reticular nucleus
DAG	4-diphenylacetoxy- <i>N</i> -methyl piperidine methiodide, a muscarinic antagonist	nRT	Oxotremorine
4-DAMP	Phenylmethyl piperazine, a nicotinic agonist	OXO	Inorganic orthophosphate
DMPP	Concentration which produces 50% of the maximum effect	$P_i$	Phosphatidate
EC <sub>50</sub>	Dose which produces 50% of the maximum effect	PA	Negative logarithm of the concentration of antagonist which shifts agonist dose-response curve two-fold to the right
ED <sub>50</sub>	Ethylenediaminetetraacetic acid	$pA_2$	Propylbenzylcholine mustard
EDTA	Ethylene glycoltetraacetic acid	PBCM	<i>p</i> -Chloromercuribenzoate, a compound which reacts with sulfhydryl groups
EGTA	Potassium equilibrium potential	PCMB	Phosphatidylinositol, a membrane lipid
$E_K$	Excitatory postsynaptic potential		Phosphatidylinositol 4-phosphate
e.p.s.p.	Guanine	PI	Phosphatidylinositol
G	Inhibitory guanine nucleotide regulatory protein	PIP	4,5-bisphosphate
G <sub>i</sub>	Stimulatory guanine nucleotide regulatory protein	PIP <sub>2</sub>	Phorbol ester; 4 $\beta$ -phorbol 12 $\beta$ -myristate 13 $\alpha$ -acetate
GABA	$\gamma$ -Aminobutyric acid	PMA	Pirenzepine, a muscarinic antagonist. Sometimes labeled with tritium ( $[^3H]$ -PZ) for binding studies
GDP	Guanosine diphosphate	PZ	Quinuclidinyl benzilate, a muscarinic antagonist. When labeled with tritium ( $[^3H]$ -QNB) used for binding studies
GppNHp	5'-guanylylimidodiphosphate nonhydrolysable analog of GTP which binds to <i>N</i> -proteins	QNB	Inhibitory cell surface receptor
H-	High affinity agonist form of the mAChR for agonists		Stimulatory cell surface receptor
HC-3	Hemicholinium-3. When labeled with tritium ( $[^3H]$ -HC-3) used for binding studies	R <sub>i</sub>	Senile dementia of the Alzheimer type
I <sub>h</sub>	Membrane potassium current gated by calcium	R <sub>s</sub>	Tetranitromethane
$I_M$ or $I_{K,M}$	Membrane potassium current controlled by muscarinic receptors	SDAT	Triphosphoinositide
IC <sub>50</sub>	Concentration which causes 50% inhibition	TNM	Tetrodotoxin, an inhibitor of nerve conduction
IP <sub>1</sub>	Inositol monophosphate	TPI	
IP <sub>2</sub>	Inositol bisphosphate	TTX	
IP <sub>3</sub>	Inositol trisphosphate		

## The molecular basis of receptor selectivity

A. S. V. Burgen

The function of drug receptors is to convert the binding of an agonist into some change in cellular activity. To accomplish this the receptor must modulate some effector system. Current evidence strongly suggests that this is accomplished by a conformational transition in the receptor. This hypothesis requires that the receptor can exist in a minimum of two conformations, a ground and excited state, although there is no necessity that we should be so restrictive. For instance, while it could be assumed that all antagonists combine with a common ground state, it is known that some antagonists such as quinuclidinyl benzilate (QNB) bind to the muscarinic receptor with two stage kinetics providing evidence of the participation of at least two conformational states relevant to binding. Further, some antagonists, notably pirenzepine, have anomalously slow kinetics incompatible with a diffusion limited reaction with the receptor. Further conformational transitions in the ground state are revealed by the complex kinetics of the gallamine-antagonist combination. It is evident that a repertoire of conformations can be called upon for binding of antagonists; functionally they represent a class that does not activate the effector.

In binding agonists, subtypes of receptor are seen that can be relatively easily interconnected by, for instance, guanine nucleotides or suitable electrolyte concentrations and can also have their binding characteristics changed by gallamine. At least some of these conformations are coupled to effectors, so that there may be a repertoire of conformations that are coupled to effectors.

There are two ways of looking at the generation of these conformations. It may be that the receptor in the absence of any ligand exists in a number of conformations which are in dynamic equilibrium, one of these conformations is complementary to the ligand and hence will be selected. If the reaction of the ligand is completely specific for that conformation, then at saturation that will be the sole conformation present. The alternative way of looking at the problem is that the ligand combines with some complementary form of the receptor but that the 'supermolecule' formed is unstable and relaxes into a new conformation that is not present in the unliganded condition; this process can be called conformation induction.

Either of these models provide for kinetics of binding that are slower than diffusion limited kinetics because they involve interconversions of conformations and the energy barrier for the interconversion becomes the determinant of the kinetics.

Since the final conformations to which antagonists and agonists bind are different, it is of some interest to know how great the differences are. The differences for the muscarinic receptors are very great and include an inversion of chirality and totally different responses to alkyl loading on the basic nitrogen. Fortunately for

medicinal chemists this is not always the case and frequently potent antagonists can be derived from structural modification of agonists. Well-known examples are the  $H_2$  antagonists in which the imidazole of the agonist, histamine, is retained, the  $\beta$ -receptor antagonists with elements of isoprenaline, and recently some potent serotonin antagonists have been found with retention of the indole. In all these cases part of the binding site has remained relatively unperturbed by the conformation change.

The receptors activate effectors either because of direct coupling on the same macromolecule or indirectly through interaction between receptor and effector subunits. Since subunit binding is formally similar to the binding of any other ligand, it may occur without perturbing the conformation of either component or may be the basis of conformation change in either or both components. What happens when a drug binds to the receptor with a change in the conformational equilibria? One possibility is that the changed receptor conformation has a different affinity for the effector subunit - this seems to be the general way in which the guanine nucleotide (N) subunits are affected in those muscarinic receptors regulating adenylate cyclase. Incidentally, guanine nucleotides seem not to change the affinity with which agonists bind the subclasses of receptor but do change the population of each subtype.

Clearly we can also have the possibility that conformation change in a receptor alters the conformation of the effector and hence its activity. We do not know at present whether this is a characteristic of any muscarinic action, but that it can occur is attested by many examples of enzymology, for instance, in aspartate transcarbamylase or between the subunits of haemoglobin.

It is clear that association of the receptor with different effector units (for cAMP,  $IP_3$ ,  $K^+$  or  $Ca^{2+}$  channels) might affect either the affinity of the receptor or its ability to respond to agonists. It is quite likely that the differences observed with certain agonists such as oxotremorine and McN-A-343 may be due to this factor, as may the differential response to pirenzepine or 4-DAMP.

However, it is premature to assume that the differences between  $M_1$  and  $M_2$  receptors are not due to sequence differences or more fundamental changes in the structure of the receptor unit. Hopefully the answer to this question will be available before long as a result of the amino acid sequencing of the receptor derived from several sources. We should recall that it is already clear that there is little or no homology in gross structure between the muscarinic and nicotinic receptors.

The existence of more than one receptor conformation that can be coupled to inactive or active states of the effector complicates the interpretation of structure-activity studies since we have to beware of concluding that drugs all belong to one structural series. The anomalies in the behaviour of the oxotremorine group compared to carbachol or acetylcholine may have this origin.

## Biochemical studies on muscarinic receptors in porcine atrium

Michael I. Schimerlik, Steve Miller, Gary L. Peterson,  
Larry C. Rosenbaum and Michael R. Tota

*Biochemical characterization of the membrane-bound, detergent-solubilized and purified muscarinic acetylcholine receptor (mAChR) from porcine atria is described. Modulation of the membrane-bound mAChR agonist binding properties by guanylyl-5'-imidodiphosphate is discussed, as are experiments with detergent-solubilized mAChRs indicating association with guanine nucleotide binding protein(s) and mutually exclusive binding of agonists and antagonists. Ligand binding and structural studies of the purified mAChR are presented. Pirenzepine appears sensitive to mAChR association with other effector proteins as opposed to discriminating between intrinsically different mAChR ligand-binding polypeptides.*

Muscarinic receptors (mAChRs) in the atria of the mammalian heart are thought to mediate several physiological responses including attenuation of adenylate cyclase activity, stimulation of inositol phospholipid metabolism, and opening of certain  $K^+$  channels.<sup>1</sup> Porcine atria may be useful for biochemical studies *in vitro* concerning the structure and mechanism of action of the atrial mAChR since tissue can be obtained in large quantities and the porcine heart and circulatory system resemble that of man.<sup>2</sup> The purpose of this communication is to present data concerning the biochemical characterization of the mAChR from porcine atria in the membrane-bound, detergent-solubilized and highly purified states.

### Membrane-bound atrial muscarinic receptors

A preparation of porcine atrial sarcolemma enriched in mAChRs by 25–40 fold<sup>3</sup> has been used to characterize muscarinic receptor ligand interactions in the membrane-bound state. Ligand binding studies on this and lower purity preparations<sup>4</sup> are consistent with a single homogeneous population of antagonist binding sites with at least two subpopulations of agonist binding sites, having high and low affinity for agonists. The data in Fig. 1A indicate that the high affinity state for agonists is coupled to a guanine nucleotide binding protein(s), since saturation with guanylyl-5'-imidodiphosphate (GppNHp) converts the high affinity state(s) to low affinity. The guanine nucleotide binding protein mediating this effect has a high affinity for GppNHp ( $K_{\text{apparent}} \sim 10$  nM, Fig. 1B). It is important to mention that the effects of guanine nucleotides are variable in this preparation, the most common observation being that 20–30% of the high affinity sites remain high affinity, even in the presence of saturating GppNHp. The variability is unaffected by the addition of leupeptin and antipain in addition to phenylmethyl sulfonyl fluoride, using higher concentrations of  $Ca^{2+}$  chelators, or adding antioxidants or

dithiothreitol. The reason for this is unknown; however, differing results from other laboratories<sup>5–7</sup> using different animals and preparative techniques lead us to speculate that this may be due to a variable amount of a subclass of high affinity agonist sites that are inherently high affinity in the absence of interaction with guanine nucleotide binding proteins or that do not decouple in the presence of GppNHp. GppNHp does not affect the binding of the tritiated antagonist 1-quinuclidinyl benzilate ( $[^3H]L$ -QNB), and positive co-operativity<sup>8</sup> of  $[^3H]L$ -QNB binding to this preparation in the presence of GppNHp was not observed.

### Detergent-solubilized atrial muscarinic receptors

Methods for solubilization of the atrial mAChR in high yields using a mixed digitonin/cholate detergent system have been developed.<sup>2,9</sup> Solubilization in phosphate buffers<sup>9</sup> generally results in conversion of the mAChR exclusively to the low affinity agonist state<sup>10</sup> suggesting an uncoupling from effectors present in the membrane while solubilization by a double extraction technique<sup>3</sup> often results in a fraction (5–20%) of mAChRs having high affinity for agonists. Using the technique of double inhibition kinetics,<sup>11</sup> it was possible to ask whether agonists and antagonists were capable of forming a ternary complex in the solubilized preparation having homogeneous agonist binding characteristics.<sup>10</sup> The scheme presented in Fig. 2A depicts a mechanism where two inhibitors ( $I_1$  and  $I_2$ ) that are competitive with a third ligand ( $[^3H]L$ -QNB, Q) can form a ternary complex with the receptor ( $RI_1I_2$ ). The mechanism for  $[^3H]L$ -QNB binding (a rapid pre-equilibrium, followed by a slow conformational change) has been derived previously for this preparation.<sup>10</sup>  $K_1$  and  $K_2$  are the dissociation constants for the two inhibitors binding to the mAChR and  $\alpha$  is a coefficient of interaction that relates the dissociation constants for the binary complexes to that for formation of the ternary complex. When the mAChR is pre-equilibrated with an excess of  $I_1$ , and the reaction is initiated by an excess of  $[^3H]L$ -QNB, the equation describing the observed rate constant ( $k_{\text{obs}}$ ) for formation of the receptor- $[^3H]L$ -QNB complex ( $RQ$ ) as a function

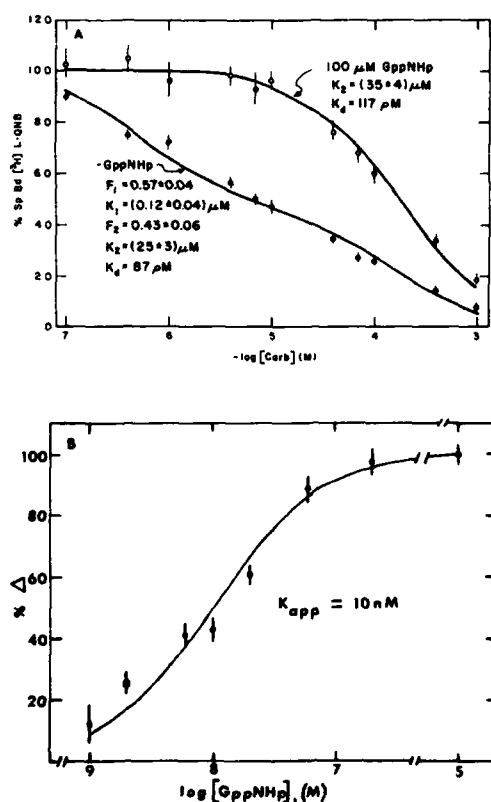


Fig. 1. Modulation of membrane-bound mAChR-agonist interactions by guanine nucleotides.

A. Carbachol titration of specifically bound  $[^3\text{H}]\text{L-QNB}$  in the presence (open circles) and absence (closed circles) of 100  $\mu\text{M}$  GppNHp. Experimental conditions (20 mM Hepes, 0.1 mM PMSF, 0.1 M NaCl, 5 mM  $\text{MgCl}_2$ , 178  $\mu\text{M}$  total  $[^3\text{H}]\text{L-QNB}$  and (mAChR), 52  $\mu\text{M}$  in  $[^3\text{H}]\text{L-QNB}$  sites. Data were analysed by the law of mass action assuming competitive inhibition at two non-interacting populations of agonist sites or simple competitive binding at a single class of sites.

B. Titration of the fractional conversion (%  $\Delta$ ) of high affinity agonist sites to low affinity by GppNHp. Conditions were similar to A, and the curve was calculated from the law of mass action assuming a single class of GppNHp binding sites with  $K_{\text{app}} = 10$  nM.

of time can be rearranged to give

$$(k_{\text{obs}} - k_-)^{-1} = \frac{K}{K_1 k_+ [Q]} \left( 1 + \frac{[I_2]}{\alpha K_2} \right) [I_1] + \frac{1}{k_+} \left( 1 + \frac{K}{[Q]} \left( 1 + \frac{[I_2]}{K_2} \right) \right)$$

When the data are plotted as  $(k_{\text{obs}} - k_-)^{-1}$  v.  $I_1$  concentration at varying  $I_2$  concentrations, it can be seen that the intercept will always be a function of  $I_2$  concentration; however, the slopes will vary with  $I_2$  only if  $\alpha$  has a finite value - the larger the value for  $\alpha$  the weaker the tendency

to form the ternary  $\text{RI}_1\text{I}_2$  complex. If  $\alpha$  is infinite, the binding of the two inhibitors is mutually exclusive, the slope will not depend on  $I_2$ , and parallel lines will be observed. The results of this experiment (Fig. 2B) done with extremely high ligand concentrations in an attempt to drive ternary complex formation, show that the agonist carbamylcholine and the antagonist *L*-hyoscyamine are mutually exclusive in their binding to the solubilized mAChR. Similar results were found for other agonist/antagonist combinations and for gallamine as well, indicating that when the mAChR is removed from the membrane environment and decoupled from possible interactions with other effectors, muscarinic ligands appear to bind in a mutually exclusive manner.

The behavior found for the mAChR solubilized using the double extraction techniques<sup>3</sup> is somewhat more complex, with a variable proportion of high affinity agonist sites appearing in the preparation (usually 20% or less). The reason for this variability is unknown, but electrophoretic studies using  $[^3\text{H}]\text{propylbenzilylcholine}$  mustard<sup>12</sup> to affinity alkylate the mAChR show relatively Gaussian distributions of label about the same apparent

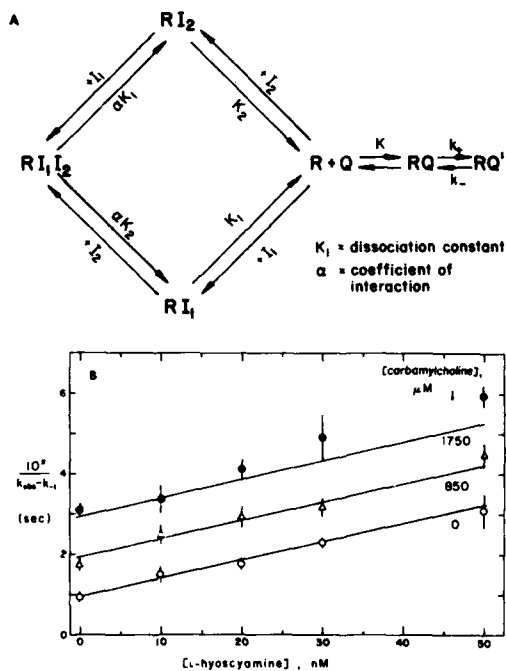


Fig. 2. Double inhibition kinetics. mAChR (0.6 nM in  $[^3\text{H}]\text{L-QNB}$  sites) was pre-equilibrated with the indicated concentrations of carbamylcholine and *L*-hyoscyamine and the reaction started by addition of 7 nM  $[^3\text{H}]\text{L-QNB}$  at time zero.  $k_{\text{obs}}$  was obtained from the slope of a semilogarithmic plot of  $\ln$  (%  $[^3\text{H}]\text{L-QNB}$  specifically bound) v. time and  $k_-$  from the experimentally determined dissociation rate constant at saturating concentrations of non-labeled L-QNB ( $2.0 \times 10^{-3} \text{ min}^{-1}$ ). Lines drawn through the data were calculated from a least squares fit to the equation in the text assuming that  $\alpha$  was infinitely large.

**Table 1.** Equilibrium dissociation constants for muscarinic ligands binding to the purified mAChR.

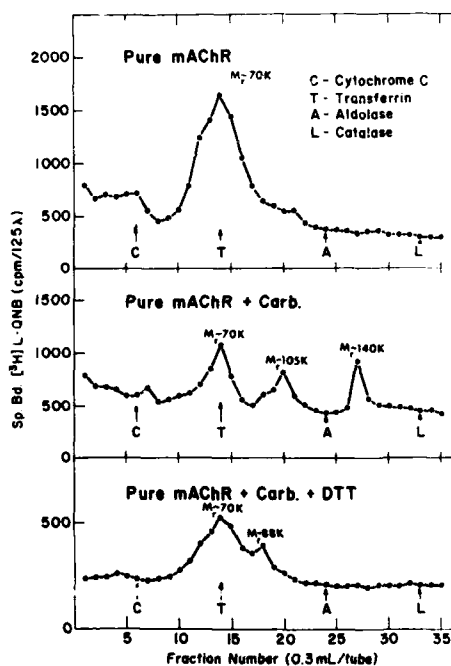
Ligand <sup>a</sup>	Dissociation constant ( <i>M</i> )	Purified <sup>b</sup>	Purified <sup>c</sup>
		membrane-bound antagonist or low affinity agonist site	detergent-solubilized
<i>Agonists</i>			
Acetylcholine <sup>d</sup>	(8.00 ± 0.53) × 10 <sup>-6</sup>	1.3 ± 0.1	0.5 ± 0.1
Carbachol	(7.14 ± 0.47) × 10 <sup>-5</sup>	1.0 ± 0.1	1.2 ± 0.2
Oxotremorine	(2.17 ± 0.14) × 10 <sup>-6</sup>	1.0 ± 0.1	0.7 ± 0.3
Acetyl-β-methylcholine <sup>d</sup>	(2.34 ± 0.15) × 10 <sup>-5</sup>	0.5 ± 0.1	not determined
<i>Partial agonist</i>			
Pilocarpine	(1.04 ± 0.10) × 10 <sup>-5</sup>	1.9 ± 0.1	8.7 ± 5.1
<i>Antagonists</i>			
1-hyoscyamine	(4.75 ± 0.03) × 10 <sup>-10</sup>	1.7 ± 0.4	not determined
Scopolamine	(8.5 ± 0.6) × 10 <sup>-6</sup>	2.9 ± 0.2	1.7 ± 0.4
Pirenzepine	(2.10 ± 0.10) × 10 <sup>-7</sup>	1.7 ± 0.2	0.4 ± 0.1 <sup>e</sup>
Dextimide	(6.05 ± 0.42) × 10 <sup>-10</sup>	0.4 ± 0.1	0.7 ± 0.1
Levetimide	(3.10 ± 0.02) × 10 <sup>-6</sup>	0.8 ± 0.1	1.5 ± 0.3
L-QNB	(6.10 ± 0.40) × 10 <sup>-11</sup>	1.5 ± 0.1	4.1 ± 0.7

<sup>a</sup> 10 mM phosphate buffer, 1 mM EDTA, 0.1 mM PMSF, pH 7.4 for all ligands unless specified otherwise<sup>b</sup> Taken from Schimerik and Searles<sup>4</sup> or determined independently in this laboratory<sup>c</sup> Taken from Herron *et al.*<sup>10</sup> or determined independently in this laboratory<sup>d</sup> pH 6.9, all other conditions the same<sup>e</sup> Low affinity site

molecular weight for the membrane-bound<sup>3</sup>, solubilized<sup>4</sup>, partially purified<sup>11</sup> and purified<sup>14</sup> preparations, indicating that this may not be due to proteolysis. Further control experiments indicate that when the high affinity agonist sites appear, the ratio of high to low affinity remains relatively constant during mAChR purification implying, but not proving, that this is not an artifact of the preparative procedure. Preliminary data indicate that the detergent extracts contain a 20 to 30 fold molar excess of the guanine nucleotide-binding proteins  $N_i$  plus  $N_o$  (substrates for ADP ribosylation by pertussis toxin<sup>15</sup>) compared to mAChR. That these proteins are capable of interacting in the solubilized state is shown in Fig. 3. Sucrose gradients in the absence of ligands show a relatively homogeneous population of mAChRs (Fig. 3, top); however, in the presence of saturating carbachol concentration, about 25% of the mAChR peak migrates to a higher apparent  $M_r$  (Fig. 3, middle). This interaction is reversed by GppNHp (Fig. 3, bottom) and by dialysis to remove carbachol (not shown). This indicates that either the mAChR population is heterogeneous in that only 25% is capable of coupling to guanine nucleotide binding protein(s) or that the equilibrium constant for the association of these two proteins under the conditions used for this experiment is greater than 200 nM, the approximate concentration of  $N_i$  plus  $N_o$  in the extract. Since this effect is reversible by dialysis to remove ligand, it seems unlikely that the association reaction is mediated by covalent bond formation. These alternatives, as well as the possibility that the mAChR may be interacting with other proteins in the detergent extract, are currently under investigation.

#### Purified atrial muscarinic receptors

The purification of the mAChR from porcine atria has been described<sup>13</sup>. The larger  $M_r$  polypeptide contains the muscarinic ligand binding site while the role of the small



**Fig. 3.** Sucrose gradients of detergent-solubilized mAChR. The gradients were run in 0.35% v/v Triton X-405 as described<sup>10</sup> and mAChR receptor determined by [<sup>3</sup>H]-L-QNB binding assay after dilution of aliquots of each fraction. Top: mAChR alone; middle, mAChR plus 10 mM carbachol; bottom, mAChR plus 10 mM carbachol and 10  $\mu$ M GppNHp.

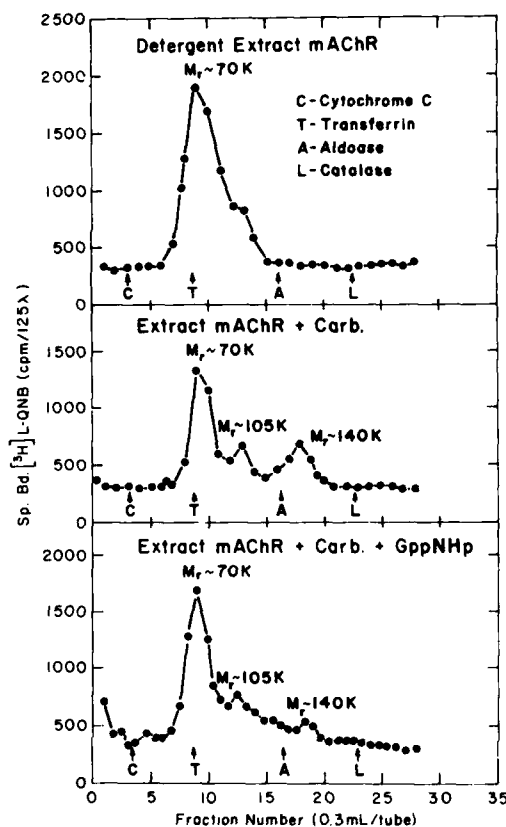
$M_r$  polypeptide is unknown; it may be a contaminant. Studies of the interactions of muscarinic ligands with the purified mAChR determined by competition v. [ $^3$ H]L-QNB are summarized in Table I. Antagonists show about the same affinity for the purified mAChR as found for the membrane-bound<sup>4</sup> and detergent solubilized preparations<sup>10</sup>. Agonist behavior is again somewhat variable. With freshly prepared mAChR in dilute solution agonists appear to interact with a single class of sites having about the same dissociation constant as the low affinity agonist sites in the membrane-bound preparation<sup>4</sup> and the detergent solubilized preparations prepared in phosphate buffer<sup>10</sup>. The presence of high affinity agonist sites (usually less than 10%, but occasionally up to 50%) is sometimes detected. The possibility of this behavior being caused by a slow detergent-induced isomerization or mAChR aggregation (see below) cannot be discounted, although for reasons described above, proteolysis does not appear to be the cause.

The purified preparation consists of two polypeptides having apparent molecular weights of about 80 and 14 kDa, respectively. The results of gel chromatography, sucrose gradients in D<sub>2</sub>O and H<sub>2</sub>O and a detailed study of the migration of the small and large  $M_r$  polypeptides by sodium dodecyl sulfate electrophoresis<sup>16</sup> are summarized in Table II. The most important result of these studies was that the large  $M_r$  polypeptide shows anomalous electrophoretic migration. Estimation of the molecular weight of the large  $M_r$  polypeptide by a novel evaluation scheme which analyses the sources of anomalous migration, gave a value of 50–60 kDa for the protein portion of the molecule<sup>16</sup>. Values of 65–70 kDa were determined for the protein-carbohydrate moiety by sucrose gradients and gel chromatography with Sephacryl S-300, after correction for bound detergent. The Stokes radius was found to be about 4.3 nm and from the frictional ratio of

**Table II.** Estimations of molecular weight, size and shape of purified mAChR.

(1) Sedimentation in sucrose gradients containing H <sub>2</sub> O or D <sub>2</sub> O (0.35% w v Triton X-405)	
(a) Partial specific volume of protein plus detergent ( $v_r$ )	0.813 cm <sup>3</sup> g
(b) Sedimentation coefficient ( $s_{20,w}$ )	$5.30 \times 10^{-13}$ s
(c) Partial specific volume of the protein ( $v_p$ )	0.717
(d) Molecular weight	
protein plus detergent ( $M_r$ )	142 600
protein alone ( $M_p$ )	70 900
(2) Gel filtration (0.35% w v Triton X-405)	
(a) Stokes radius	42.9 Å
(b) Molecular weight:	
protein plus detergent	116 300
protein alone	58 200
(c) Frictional ratio ( $F/F_r$ )	1.21
(3) SDS-PAGE experiments	
(a) Average $M_r$ from $^{20}$ S-T (range 6–17%)	$83\,300 \pm 10\,400$
(b) $M_r$ from Ferguson plots	$89\,000 \pm 6700$
	$14\,000 \pm 1100$
(c) $M_r$ from analysis of abnormal values of size and charge	$55\,000 \pm 5000$
	$14\,300$

<sup>a</sup> Low molecular weight peptide found in purified preparation.



**Fig. 4.** Sucrose gradient of purified mAChR. Conditions are similar to Fig. 3. Top, mAChR alone, middle, mAChR plus 10 mM carbachol, bottom, mAChR plus 10 mM carbachol and 1 mM dithiothreitol.

1.21, the purified mAChR appeared to behave as a hydrated, globular protein in the presence of Triton X-405. Sucrose gradients (Fig. 4) indicate that in the absence of ligands, or in the presence of antagonists relatively homogeneous migration ( $M_r \sim 70$  kDa) is found (Fig. 4, top); however, in the presence of the agonist carbachol, a high  $M_r$  peak corresponding to a dimer ( $M_r \sim 140$  kDa) is observed (Fig. 4, middle). Treatment with the reducing agent dithiothreitol eliminates the high  $M_r$  peak (Fig. 4, bottom). At this time it is not known whether this is due to reduction of an intra- or intermolecular disulfide bond; however, it is tempting to speculate that, by analogy with the detergent extract, carbachol binding induces a conformational change in about 25% of the mAChRs exposing a hydrophobic surface. Hydrophobic interactions can then cause association, either with a guanine nucleotide binding protein in the case of the detergent extract, or with another mAChR molecule in the purified preparation. Reduction of an

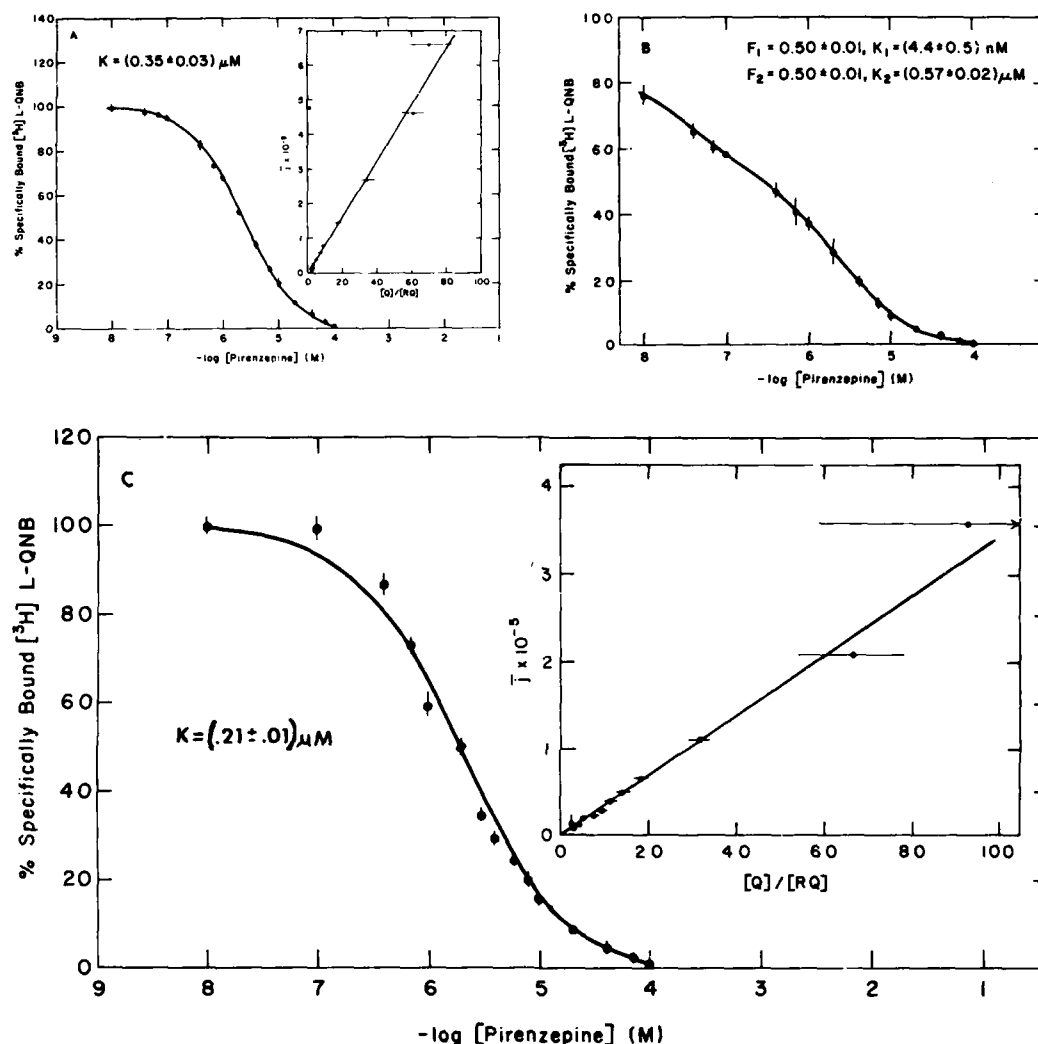


Fig. 5. Pirenzepine titrations of membrane-bound digitonin cholate-solubilized and purified mACHR.

A. Membrane-bound mACHR. mACHR (156 pmol in [ $^3$ H]L-QNB sites), 250 pmol [ $^3$ H]L-QNB, 10 mM potassium phosphate, 1 mM EDTA, 0.1 mM PMSE, pH 7.4. Data were analysed using a weighted linear least squares fit to the  $\bar{y}$  function as previously described.<sup>4</sup>

B. Detergent-solubilized mACHR. Same buffer as A. mACHR was 388 pmol in [ $^3$ H]L-QNB sites and total [ $^3$ H]L-QNB equalled 590 pmol. Data were analysed assuming competitive inhibition at two non-interacting subclasses of [ $^3$ H]L-QNB binding sites.<sup>4</sup>

C. Purified mACHR. Same buffer as A. mACHR was 202 pmol in [ $^3$ H]L-QNB sites and total [ $^3$ H]L-QNB equalled 550 pmol. Data were analysed as in A.

intramolecular disulfide bond alters the receptor conformation, preventing the association from occurring.

#### Muscarinic subtypes: interactions with pirenzepine

Pirenzepine (PZ) is a unique muscarinic antagonist able to differentiate between  $M_1$  (high affinity) and  $M_2$  (low affinity) muscarinic subtypes<sup>17</sup>. Pirenzepine interactions with the membrane-bound, solubilized and highly purified

mACHR are shown in Fig. 5. In the membrane-bound preparation (Fig. 5A), PZ appears to displace [ $^3$ H]L-QNB from a homogeneous population of binding sites having low affinity for PZ ( $K_i = 0.35 \mu\text{M}$ ) in agreement with the classification of atrial muscarinic receptors as  $M_2$ . In the solubilized state (Fig. 5B), however, about 50% of the [ $^3$ H]L-QNB binding sites remain in the low affinity state ( $K_i = 0.57 \mu\text{M}$ ) but 50% of the sites exhibit high affinity for

PZ ( $K_H=4.2$  nM). The purified mAChR population (Fig. 5C) shows only low affinity PZ binding ( $K_i=0.21$   $\mu$ M). The simplest interpretation of these data, is that upon solubilization and removal from the constraints of the membrane, the mAChR is free to associate with other proteins of unknown identity which then cause a subtle alteration of mAChR binding properties. This alteration must be subtle, since little or no difference is found for the dissociation constants of other ligands between the membrane-bound and solubilized states (Table I). Alternatively, PZ binding to solubilized mAChR could induce a unique conformation that permits interactions with other proteins. Removal of the non-receptor proteins upon purification prevents these interactions from taking place, giving rise to a homogeneous population of low affinity PZ sites. Thus, PZ does not appear to recognize distinct classes of the mAChR ligand binding protein *per se*, but does appear to be an exquisitely sensitive probe for the interaction of the receptor polypeptide with other effector proteins.

#### Summary

Biochemical studies on the membrane-bound, detergent solubilized and highly purified mAChR from porcine atria have been described. Much of the data presented is clearly preliminary; however, the results support the notion that the system is a complex one in which mAChRs may be capable of interacting with each other, as well as other effector proteins. Furthermore, differing ligands may either induce different mAChR conformations or detect subtle differences in mAChR-effector interactions. Determining the nature and specificity of these interactions is a challenging problem for future research efforts.

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## Muscarinic receptor subclasses: the binding properties of the soluble receptor binding sites

Christopher P. Berrie, Nigel J. M. Birdsall,  
Edward C. Hulme, Mary Keen,  
Jane M. Stockton and Mark Wheatley

*Muscarinic receptor subclasses from the rat cerebral cortex, myocardium and lacrimal gland have been solubilized in digitonin in a stable form. The pattern of selectivity of pirenzepine in distinguishing between the solubilized muscarinic receptor subclasses is different from that found in membranes. In solubilized preparations from the myocardium, agonist binding is complex. This is caused by the presence of receptor-GTP binding protein complex which has a higher sedimentation coefficient (13.4 S) than the apparently monomeric receptor (11.6 S). The purified cortical muscarinic receptor can exhibit complex binding properties for agonists and pirenzepine. It is concluded that differences in the properties of the muscarinic receptor subclasses are retained on solubilization of the receptor in digitonin.*

There is now strong evidence from both pharmacological and biochemical studies that there are subclasses of muscarinic receptors (for reviews see, for example, Refs 1-4). There are antagonists such as 4-diphenylacetoxy-N-methylpiperidine methiodide,<sup>5</sup> hexahydroisiladifenidol<sup>6</sup> and secoverine<sup>7</sup> which selectively block muscarinic responses in smooth muscle whilst having weaker actions on the heart. Gallamine<sup>8</sup> and some other neuromuscular blockers<sup>4</sup> have, in contrast, a cardioselective action which appears to result from their binding to a second binding site on muscarinic receptors.<sup>9,10</sup> However, the drug which has provided the major impetus in focusing research on the existence and nature of muscarinic receptor subclasses is pirenzepine (PZ), which is a muscarinic antagonist used in the treatment of peptic ulcer disease. It selectively inhibits vagally stimulated gastric secretion and various neuronal muscarinic responses, but has a lower potency for heart and smooth muscle receptors.<sup>2,3</sup> The selectivity found in functional whole tissue assays is also present in biochemical assays of receptor function<sup>11,14</sup> and when the PZ binding properties of muscarinic receptors in membrane preparations from different tissues are examined (see, for example, Refs 1, 2, 4, 15).

The origin of the selective actions of PZ (and other antagonists) is still unknown. There are two basic explanations: the differences in receptor subtypes may reflect either an intrinsic difference in the structure of the receptor or a difference in the environment of the receptor. In the first category are receptor isotypes (different amino acid sequences) or receptors with the same sequence but which have been subjected to different tissue-specific post-translational modifications (e.g. glycosylation, phosphorylation, acylation). In the second category are differences in the coupling to effector(s)<sup>12</sup> or in the structure and composition of the membrane.<sup>16</sup> These categories are not mutually exclusive: it may be

possible that a structural change in the receptor will favour the coupling of a receptor molecule to one of several potential effector molecules which are present in the same locale. For example, the factors which determine the interaction of certain receptors with one (or more) of

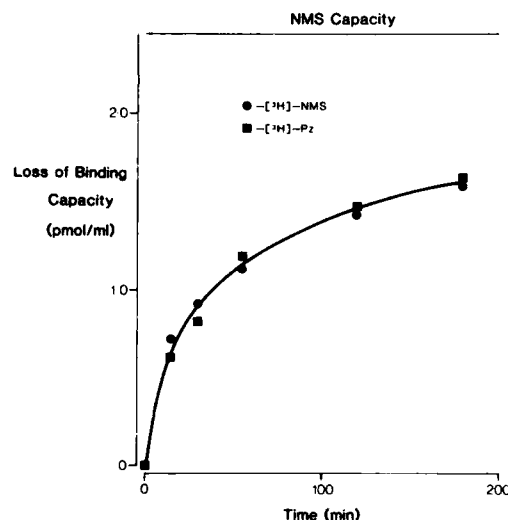


Fig. 1. Loss of [<sup>3</sup>H]-PZ and [<sup>3</sup>H]-NMS binding sites in solubilized cortical preparations at 30 °C. The soluble preparations were incubated at 30 °C (without radioligands) for the specified times and then cooled to 0 °C to quench the loss of receptor sites. [<sup>3</sup>H]-NMS (30 nM) and [<sup>3</sup>H]-PZ (3 nM) were added and incubation continued for 40 h at 4 °C. The [<sup>3</sup>H]-NMS bound reflects the total number of receptor binding sites. The number of [<sup>3</sup>H]-PZ binding sites was calculated using the affinity constant for high affinity PZ binding sites ( $6.5 \times 10^7 \text{ M}^{-1}$ ) and was 1.99 pmoles/ml at zero time. Data taken from Ref. 28.

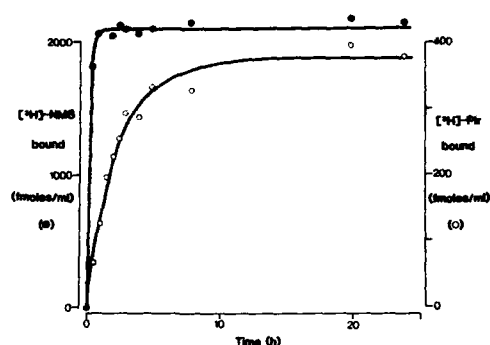


Fig. 2. Time course of receptor specific binding of  $[^3\text{H}]\text{-NMS}$  and  $[^3\text{H}]\text{-PZ}$  binding to a soluble cortical preparation. The concentration of both radioligands was ( $10^{-8}$  M). The rate constant for  $[^3\text{H}]\text{-PZ}$  ( $[^3\text{H}]\text{-Pir}$ ) binding was  $0.45\text{ h}^{-1}$  and that for  $[^3\text{H}]\text{-NMS}$   $4\text{ h}^{-1}$ . The figure is reproduced from Ref. 28 with permission.

the ever increasing family of GTP-binding proteins ( $N$  proteins, e.g.  $N_1$ ,  $N_2$ ,  $N_3$ , transducin) are currently the subject of intense research.

One of the ways in which we have started to examine these possibilities is by determining the binding properties of solubilized muscarinic receptors from different tissues. By this means, we aim to eliminate the influence of the membrane environment on the binding properties and to investigate the possibility of solubilizing and characterizing specific receptor-effector complexes.

The tissues selected are the rat cerebral cortex, myocardium and lacrimal gland. The muscarinic receptors in these tissues exhibit different agonist and antagonist binding properties and appear to be coupled to several effector mechanisms.

In the cerebral cortex, there are both high and low affinity PZ binding sites;<sup>15</sup> the former sites can be labelled directly with  $[^3\text{H}]\text{-PZ}$  (Refs 17-22). The binding of agonists to the high affinity PZ binding sites is modulated by divalent ions (e.g.  $\text{Mg}^{2+}$ ) and guanine nucleotides whereas the low affinity sites for PZ in our membrane preparations do not appear to be capable of such coupling and uncoupling to GTP binding proteins.<sup>17</sup> As regards function, Gil and Wolfe<sup>13</sup> have reported that in the rat forebrain, PZ more potently antagonizes the action of muscarinic agonists to stimulate breakdown of inositol phospholipids, than to inhibit adenylate cyclase.

In the myocardium, muscarinic receptors predominantly have a low affinity for PZ (Ref. 15) and their agonist binding properties are strongly modified by  $\text{Mg}^{2+}$  and guanine nucleotides (e.g. Ref. 23). Muscarinic receptor mediated inhibition of adenylate cyclase can be demonstrated readily in membranes<sup>24,25</sup> and in whole tissue but it has also been possible to demonstrate a stimulation of triphosphoinositide (TPI) turnover.<sup>13,26</sup>

Muscarinic receptors in the lacrimal glands have an intermediate affinity for PZ (Ref. 15), and their agonist binding properties are only affected by guanine nucleotides to a minor extent. The receptors in single cell preparations from this tissue appear to be very efficiently coupled to TPI turnover (Berrie, C. P., unpublished results).

Some care was taken in the preparation of the membranes and the choice of conditions for the solubilization and the binding assay. Membranes were treated with KCl/pyrophosphate and EDTA to remove some extrinsic proteins (including some proteases), increase specific/non-specific binding of  $[^3\text{H}]\text{-ligands}$  and to facilitate the ability of the receptors to interact with  $N$ -proteins.<sup>23</sup> Solubilization was carried out at 0°C using digitonin, and the binding assays were carried out at 4°C in a buffer containing 20 mM NaHepes, 1 mM  $\text{Mg}^{2+}$ , 1% digitonin, pH 7.5 (Ref. 27, 28). These conditions give a stable, soluble receptor preparation and, as will be shown later, allow the detection of both the monomeric binding protein and the receptor- $N$  protein complexes.<sup>27</sup> Binding studies on membranes were carried out in the same buffer less digitonin.<sup>23</sup>

#### High and low affinity soluble pirenzepine binding sites from cerebral cortex

In cortical membranes, 50-80% of the muscarinic receptors have a high affinity for PZ ( $K_A \sim 10^8\text{ M}^{-1}$ ) and can be labelled by  $[^3\text{H}]\text{-PZ}$  (Ref. 11, 17-22). The remaining sites have a considerably lower affinity ( $1-1.5 \times 10^9\text{ M}^{-1}$  under these conditions).<sup>28</sup> The high affinity sites could be solubilized by digitonin in 30-35% yield which was somewhat lower than the overall yield of binding sites (35-40%) estimated with  $[^3\text{H}]\text{-N-methylscopolamine}$ ,  $[^3\text{H}]\text{-NMS}$ .<sup>28</sup> Solubilization yields were comparable whether the receptors were labelled with  $[^3\text{H}]\text{-ligands}$  before or after solubilization. Unsolubilized binding sites were recovered undenatured in the pellet.

When the solubilization was carried out at 30°C (30 min), up to 85% of the total sites but only 35% of the  $[^3\text{H}]\text{-PZ}$  binding sites were recovered in the supernatant. From an examination of unsolubilized receptors, it was found that there was a 40-50% loss in overall  $[^3\text{H}]\text{-PZ}$  binding.<sup>28</sup>

The soluble binding sites are very stable at 4°C, less than 15% of  $[^3\text{H}]\text{-NMS}$  and  $[^3\text{H}]\text{-PZ}$  binding being lost over 24 h. However, receptors solubilized at 4°C, were unstable at 30°C in the absence of a ligand. The number of high affinity soluble PZ binding sites, affinity  $6.5 \times 10^7\text{ M}^{-1}$ , decreased in parallel with the number of  $[^3\text{H}]\text{-NMS}$  sites (Fig. 1). This suggests that the loss in binding at 30°C is caused by a selective degradation of the subpopulation of receptor binding sites which have a high affinity for PZ. This conclusion differs from that of Roeske and Venter<sup>29</sup> who suggested that high affinity PZ binding sites represent a state of the receptor which undergoes an allosteric conformational change in solution and is converted to low affinity sites. It should be noted that different receptor conformations may have different protease susceptibilities. It is therefore possible that the effects of proteolysis could be different under the two experimental conditions.

The soluble binding sites labelled by  $[^3\text{H}]\text{-PZ}$  exhibited a pharmacology entirely in agreement with their being a subpopulation of the total muscarinic receptor binding sites. These sites, of affinity  $6.5 \times 10^7\text{ M}^{-1}$ , constituted about 60% of the total sites. The lower affinity sites, analysed from a  $[^3\text{H}]\text{-NMS/PZ}$  competition experiment, had an affinity of  $\sim 4 \times 10^9\text{ M}^{-1}$ , apparently slightly higher than that found in membranes.<sup>28</sup>

One notable feature of the binding of  $[^3\text{H}]\text{-PZ}$  to soluble cortical receptors was the very slow rate of binding

( $K_d = 0.5 \text{ h}^{-1}$ ,  $10 \text{ nM}$ ) compared to  $[^3\text{H}]\text{-NMS}$  ( $\sim 4 \text{ h}^{-1}$ ,  $10 \text{ nM}$ ) (Fig. 2). This necessitated long incubations to allow equilibrium to be reached (24 h at  $4^\circ\text{C}$ ) but had the advantage that, because of the corresponding very slow off-rate, it was possible to characterize the  $[^3\text{H}]\text{-PZ}$  binding sites by sucrose density centrifugation.

The  $[^3\text{H}]\text{-PZ}$  labelled receptor sedimented as a sharp, apparently monodisperse peak of  $11.8 \text{ S}$  (Ref. 28), which is the same  $S$  (sedimentation coefficient) value as the somewhat broader and asymmetric peak of  $[^3\text{H}]\text{-NMS}$ ,  $[^3\text{H}]\text{-quinuclidinyl benzilate}$  and  $[^3\text{H}]\text{-propylbenzylcholine mustard}$  labelled receptors.<sup>30</sup> Although in membranes the receptor binding sites labelled with  $[^3\text{H}]\text{-PZ}$  are capable of coupling to a guanine nucleotide binding protein,<sup>17</sup> there was no evidence of such an interaction when the unliganded receptor was solubilized. First, agonist binding to the  $[^3\text{H}]\text{-PZ}$  binding site was of low affinity and unaffected by guanine nucleotides. Second, there was no evidence of a  $13.4 \text{ S}$  component of the sucrose density gradient profile, characteristic of the muscarinic receptor- $N$  protein complex found in the heart and described later in this paper. However, it was possible to prelabel the receptor in the membrane with the potent agonist  $[^3\text{H}]\text{-oxotremorine-M}$  and to solubilize a  $13.4 \text{ S}$  component of  $[^3\text{H}]\text{-oxotremorine-M}$  binding which was sensitive to guanine nucleotides. It was not possible to solubilize by this protocol a cortical receptor- $N$  protein complex in the absence of agonist. Therefore the unliganded cortical receptor- $N$  protein complex(es) appear to be somewhat less stable than those found in the myocardium.<sup>27</sup>

The major finding of this study is thus that cortical muscarinic receptors having both high and low affinity for PZ can be solubilized in digitonin with relatively little change in affinity.

#### Digitonin-solubilized receptor binding sites from the myocardium

Digitonin (1%) solubilized about 45% of the myocardial muscarinic receptors. The affinity of  $[^3\text{H}]\text{-NMS}$  for soluble myocardial muscarinic receptors was  $4 \times 10^9 \text{ M}^{-1}$ , a value comparable to those found in membranes in the absence ( $3 \times 10^9 \text{ M}^{-1}$ ) and presence ( $6 \times 10^9 \text{ M}^{-1}$ ) of the GTP analogue 5'-guanylylimidodiphosphate ( $10^{-4} \text{ M}$ ). This latter behavior, that is, guanine nucleotide sensitive binding of antagonists to myocardial membrane-bound muscarinic receptors has been described elsewhere.<sup>31,32</sup>

Under these experimental conditions, PZ binding to membranes, as determined from a  $[^3\text{H}]\text{-NMS/PZ}$  competition experiment, was of low affinity. The inhibition curve was slightly flatter than a simple mass action curve. The major population of binding sites had an affinity of  $\leq 10^6 \text{ M}^{-1}$  and there was no evidence of sites of  $10^8 \text{ M}^{-1}$  from a two-site analysis of the inhibition curve. However, after solubilization there was a dramatic increase in PZ binding (measured by a  $[^3\text{H}]\text{-NMS/PZ}$  competition experiment), the  $\text{IC}_{50}$  value decreasing by 20 fold. As found in the membranes, PZ binding was somewhat heterogeneous. Analysis of the inhibition curve according to a two-site model suggested that about 50% of the sites had a high affinity ( $4 \times 10^7 \text{ M}^{-1}$ ), the other half being of lower affinity ( $4 \times 10^6 \text{ M}^{-1}$ ); the lower affinity value was nevertheless higher than that found in membranes.

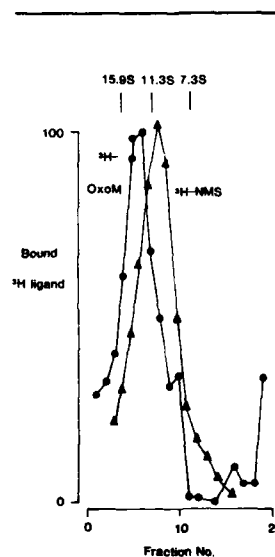
In accord with this analysis it was possible to label the

soluble myocardial receptors with  $[^3\text{H}]\text{-PZ}$ . A 60% subpopulation of the soluble muscarinic receptors bound  $[^3\text{H}]\text{-PZ}$  with an affinity of  $3 \times 10^7 \text{ M}^{-1}$ . This binding component was inhibited by nanomolar concentrations of atropine and NMS, demonstrating its muscarinic nature. It, therefore, appears that the increase in PZ affinity is a result of the release of a membrane constraint on the receptor.

$[^3\text{H}]\text{-PZ}$  binding to the soluble myocardial receptor was extremely slow ( $0.05 \text{ h}^{-1}$ ,  $10 \text{ nM}$ ), in fact, ten times slower than that found for the cortical receptor.<sup>28</sup> Hence very long incubation times (48–72 h) were necessary for equilibrium to be approached. As expected, the off-rate was very slow ( $t_{1/2}$  24 h), and this allowed us to show that the  $S$  value of the  $[^3\text{H}]\text{-PZ}$  binding site was 11.1 (Fig. 3), very similar to that found for the  $[^3\text{H}]\text{-NMS}$  labelled receptor (11.6  $S$ ) (Ref. 27). The  $[^3\text{H}]\text{-PZ}$  labelled peak was only very slightly asymmetric and had a peak width only 25% greater than the catalase standard. It would therefore appear that, as in the cortex, high affinity  $[^3\text{H}]\text{-PZ}$  binding is associated with a monodisperse and probably monomeric muscarinic receptor species.

In contrast to the behavior manifest by the soluble high affinity PZ sites in the cortex, agonist inhibition of  $[^3\text{H}]\text{-PZ}$  binding was heterogeneous and affected by guanine nucleotides. In the absence of 5'-guanylylimidodiphosphate (GppNHp), a binding site with an affinity of  $> 10^8 \text{ M}^{-1}$  for oxotremorine-M could be discerned together with a site of much lower affinity ( $10^4 \text{ M}^{-1}$ ). Binding to the high affinity sites was inhibited by GppNHp. The same phenomenon has been observed in  $[^3\text{H}]\text{-NMS/oxotremorine-M}$  competition experiments.<sup>27</sup> In fact it has been possible to label up to 60% of soluble myocardial muscarinic receptors with  $[^3\text{H}]\text{-oxotremorine-M}$  (affinity  $c. 10^9 \text{ M}^{-1}$ ) and to show that the sites sediment

Fig. 3. Sucrose density gradient centrifugation of soluble monomeric and complexed muscarinic receptors. Soluble myocardial membranes were incubated with either  $[^3\text{H}]\text{-oxotremorine-M}$  ( $10 \text{ nM}$ ) ( $\bullet$ ) or  $[^3\text{H}]\text{-N-methylscopolamine}$  ( $\blacktriangle$ ) ( $10 \text{ nM}$ , in the presence of 5'-guanylylimidodiphosphate,  $10^{-4} \text{ M}$ ) for 2 h at  $0^\circ\text{C}$ , to generate complexed and uncomplexed receptors respectively. The membranes were solubilized in digitonin, and then subjected to sucrose density gradient centrifugation as described elsewhere.<sup>27</sup> The appropriate radioligand was present in the gradient at  $10 \text{ nM}$  concentrations to increase recovery of labelled receptors. Galactosidase ( $15.9 \text{ S}$ ), catalase ( $11.3 \text{ S}$ ) and lactate dehydrogenase ( $7.3 \text{ S}$ ) were used as internal markers.



at 13.4 S (Ref. 27) (Fig. 3). In the presence of GppNHp this 13.4 S component cannot be detected with [ $^3$ H]-NMS or [ $^3$ H]-oxotremorine-M. We have postulated that the 13.4 S species with a high affinity for agonists, represents a complex between the ligand binding subunit of the receptor and a guanine nucleotide binding protein.<sup>27</sup> The evidence is that, although [ $^3$ H]-PZ-myocardial receptor complex is apparently monomeric, the receptor site is capable of interacting with an *N*-protein.

It is clear, therefore, that the solubilization of the myocardial receptor greatly reduces, but probably does not eliminate completely,<sup>33</sup> the ability of PZ to discriminate between myocardial and cortical receptors (Fig. 4). This would argue that the binding domains on the receptors are very similar<sup>16,34,35</sup> but that there are differences in the membrane environment. Nevertheless, PZ binding heterogeneity persists in solubilized preparations from a given tissue and differences between the receptors persist, especially with regard to the kinetics and the ability of the receptors to couple to *N*-proteins. Furthermore, a 4–5 fold cardioselective allosteric interaction of gallamine with muscarinic receptors persists on solubilization.

There are a number of recent reports on the selectivity of PZ which can be compared with our results on the soluble myocardial and cortical receptors. First, high affinity PZ binding sites have been found in the embryo chick heart membranes<sup>14</sup> suggesting that the receptors in this preparation may not be subject to the same membrane constraint as found in the rat heart. Secondly, in whole chick cardiac cells Brown *et al.*<sup>14</sup> found a selective action of PZ in reversing muscarinic agonist inhibition of adenylate cyclase ( $K_i = 48$  nM) compared to its inhibition of the TPI response ( $K_i = 260$  nM). In contrast, Gil and Wolfe<sup>13</sup> found in the forebrain the reverse selectivity for adenylate cyclase ( $K_i = 210$  nM) and TPI turnover ( $K_i = 21$  nM). The  $K_i$  values reported in the two papers are very close to the dissociation constants for the high and low affinity PZ binding sites in the soluble myocardial and cortical receptors. These functional observations would suggest that muscarinic receptors with different affinities for PZ can be discriminated in biochemical functional tests but, surprisingly, the receptor subclass does not appear to determine the nature of the biochemical response.

#### Digitonin solubilized receptor binding sites from the lacrimal gland

Digitonin (1%) solubilized 20–30% of the total binding sites in membranes prepared from the rat lacrimal gland. As with the other tissues, the affinity of [ $^3$ H]-NMS was relatively unchanged ( $4 \times 10^9$  M<sup>-1</sup>) and was virtually identical to that found in solubilized receptors from other tissues. PZ binding to the membrane-bound receptors, as determined from a [ $^3$ H]-NMS/PZ competition, experiment was to a uniform population of low affinity sites ( $K_i = 9 \times 10^6$  M<sup>-1</sup>). Most surprisingly, on solubilization PZ binding decreased about 6 fold. This behaviour is different from that observed in the tissues described previously and also in the medulla-pons where high affinity PZ binding is generated on solubilization (Keen, M., unpublished results).

We considered the possibility that there was a factor present in the soluble lacrimal preparation which was

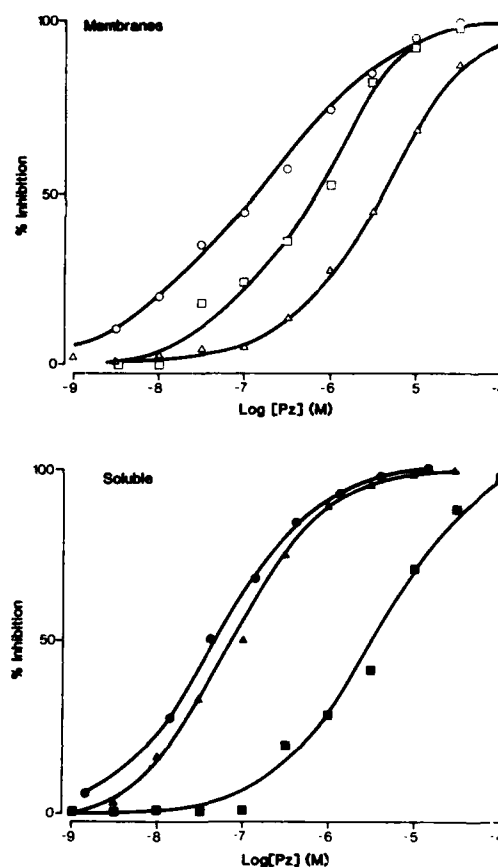


Fig. 4. PZ/[ $^3$ H]-NMS competition experiments on membrane-bound (open symbols) and soluble muscarinic receptors (closed symbols) from the cerebral cortex (circles) lacrimal gland (squares) and myocardium (triangles) of the rat. The experiments were carried out at 4°C using 0.2 nM [ $^3$ H]-NMS and the protocol described in Ref. 28. The curves are simple mass action curves (lacrimal) and best fit two-site curves (cortex and heart) and are not corrected for occupancy by [ $^3$ H]-NMS.

responsible for reversibly or irreversibly (e.g. by proteolysis) changing the binding of PZ. However, co-solubilization of cortical and lacrimal membranes gave a [ $^3$ H]-NMS/PZ inhibition curve which was precisely that expected from the proportions of high and low affinity PZ binding sites present in control unmixed receptor preparations.

These preliminary results suggest that the differences between lacrimal and cortical/myocardial muscarinic receptors are amplified by solubilization (Fig. 4).

#### Binding properties of purified muscarinic receptor preparations

The purification and binding properties of muscarinic receptors from porcine heart,<sup>36</sup> brain<sup>37</sup> and rat cortex<sup>38</sup>

have been reported. The purifications from porcine tissue utilized digitonin/cholate as detergent whilst our purification used digitonin. In all cases the predominant broad band at 65-80 K Daltons was seen on SDS gel electrophoresis (Laemmli system) as was observed by Andre *et al.*<sup>36</sup> Haga and Haga<sup>37</sup> found uniform low affinity agonist and PZ binding to their pure receptor whereas Peterson *et al.*<sup>36</sup> found heterogeneous agonist binding and did not report on the PZ binding properties of their pure receptor. We found that during purification of the cortical receptors, both the high and low affinity PZ binding sites bind and coelute from the DEAE-Sephacel column and are both taken up on the affinity column.<sup>40</sup> However, predominantly low affinity sites are eluted from the affinity column. This may result from the selective instability of high affinity PZ sites caused by proteolysis or denaturation. As a result, after two rounds of affinity chromatography, the purified receptor is predominantly (>75%) of low affinity for PZ but in some preparations, a low percentage of high affinity PZ binding can be detected either from a [<sup>3</sup>H]-NMS/PZ competition experiment or directly with [<sup>3</sup>H]-PZ. Agonist binding to the purified rat cortical receptor was also heterogeneous, if carbachol was used to elute the receptors from the affinity gel, despite the fact that in the original solubilized preparation agonist binding was of low affinity.<sup>28</sup> The high affinity agonist binding seen in our purified preparations is not sensitive to guanine nucleotides. We do not know at present whether the complex agonist binding is a result of exposure of the receptor to high concentrations of agonist resulting in either formation of a receptor-protein complex or modification of the receptor. We do not detect protein bands at 35 K and 39 K, characteristic of *N*-proteins. Nevertheless, it is of interest that in all three receptor preparations<sup>36-38</sup> there is a peptide of 12-15 000 molecular weight which appears to copurify with the receptor. Whether or not this protein is important for receptor function remains to be determined.

The availability of pure receptor in amounts sufficient to obtain amino acid sequence information and anti-receptor antibodies means that, using molecular genetic techniques, the differences (if any) in the primary amino acid sequence of the muscarinic receptor subtypes will soon be available.

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## Characteristics of two biochemical responses to stimulation of muscarinic cholinergic receptors

T. K. Harden, L. I. Tanner, M. W. Martin, N. Nakahata, A. R. Hughes, J. R. Hepler, T. Evans, S. B. Masters and J. H. Brown

*Two prominent responses occur as a result of activation of muscarinic cholinergic receptors. In some tissues the primary response is an inhibition of adenylate cyclase activity while in others phosphoinositide turnover is enhanced with consequent increases in diacylglycerol and cytoplasmic  $Ca^{2+}$  concentrations. The salient features of each of these biochemical responses will be reviewed and results from our laboratories involving two model systems that have allowed delineation of several unique characteristics of each response will be discussed.*

### Muscarinic receptor-mediated inhibition of adenylate cyclase

The initial observation by Murad *et al.*<sup>1</sup> that acetylcholine inhibits adenylate cyclase was eventually confirmed for muscarinic and other receptors, e.g.  $\alpha_2$ -adrenergic, opiate, and adenosine receptors in the mid-to-late 1970's.<sup>2</sup> All of these receptors apparently inhibit adenylate cyclase through the same mechanism. (See Fig. 1.)

Hormone responsive adenylate cyclase is composed of at least five types of components: the catalytic protein (C), stimulatory ( $G_s$ ) and inhibitory ( $G_i$ ) guanine nucleotide regulatory proteins, and stimulatory ( $R_s$ ) and inhibitory ( $R_i$ ) cell surface receptors.<sup>3</sup> Both regulatory proteins consist of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits. The  $\alpha$ -subunits of  $G_s$  and  $G_i$  are structurally similar but functionally distinct proteins, while the 35 000 M<sub>r</sub>  $\beta$ -subunits of  $G_s$  and  $G_i$  are apparently identical. Little is known about the structure and function of the  $\gamma$ -subunits. The  $\alpha$ -subunit of each regulatory protein binds GTP and is the substrate for the action of a bacterial toxin. In the presence of NAD, cholera toxin ADP-ribosylates the  $\alpha$ -subunit of  $G_s$ , while pertussis toxin ADP-ribosylates the  $\alpha$ -subunit of  $G_i$ .

In simplified terms, hormonal stimulation of adenylate cyclase occurs when a hormone interacts with  $R_s$ , e.g. the interaction of adrenaline with the  $\beta$ -adrenergic receptor, resulting in an exchange of GTP for GDP on the  $\alpha$ -subunit of  $G_s$ , dissociation of  $G_s$  into  $GTP\alpha + \beta\gamma$ , and formation of an active enzymatic species,  $GTP\alpha \cdot C$  (Ref. 3). It has been proposed that adenylate cyclase activity is returned to the ground state as a result of a GTPase activity associated with  $\alpha$ . Inhibition of adenylate cyclase by muscarinic and other inhibitory receptors involves hormone receptor-stimulated binding of GTP to  $G_i$  which in turn dissociates into  $GTP\alpha + \beta\gamma$ . Studies with resolved components indicate that  $\beta\gamma$  can decrease the activity of C by reassociating with activated  $\alpha$ . A considerable excess of  $G_i$  over  $G_s$  in target tissues favors

this mechanism. An alternate mechanism of inhibition of adenylate cyclase proposes direct inhibition of C by  $GTP\alpha$  (Ref. 4).

### Muscarinic receptor-mediated stimulation of phosphoinositide breakdown

Muscarinic receptors and other hormone receptors that mobilize  $Ca^{2+}$  also increase the turnover of phosphoinositides. In 1975 Michell<sup>5</sup> proposed that there was more than a casual relationship between these two effects and that indeed, phosphoinositide breakdown catalysed by a phospholipase C was a necessary antecedent to  $Ca^{2+}$  mobilization. Subsequently, it has been demonstrated that the polyphosphoinositide, phosphatidylinositol 4,5-bisphosphate ( $PIP_2$ ) is the key substrate in this hormone action and that the inositol phosphate product of its hydrolysis, inositol 1,4,5-trisphosphate ( $IP_3$ ), is a second messenger that mobilizes intracellular  $Ca^{2+}$  (Ref. 6). The mechanism whereby activation of muscarinic and other receptors results in an increase in the activity of a membrane phospholipase C is not known. Indeed, it has not been proven unambiguously that enzyme activity *per se*, rather than substrate access, is increased as a result of receptor stimulation. It is clear, however, that receptor activation leads to the hydrolysis of  $PIP_2$  to diacylglycerol (DAG) and  $IP_3$ . Both products serve as 'second messengers'. DAG activates a calcium, phospholipid-sensitive protein kinase called protein kinase C (Refs 6, 7). This kinase is not activated by  $Ca^{2+}$ -calmodulin or by cyclic AMP, nor does it, in general, catalyse the phosphorylation of the protein substrates for the kinases that are activated by  $Ca^{2+}$ -calmodulin or cyclic AMP.  $IP_3$  subserves a very different function in hormone action. That is, this second messenger mobilizes  $Ca^{2+}$  from non-mitochondrial storage sites, particularly those of the endoplasmic reticulum. (See Fig. 1.)

### Muscarinic receptors of astrocytoma cells

Gross and Clark<sup>8</sup> reported in 1977 that cholinergic stimuli reduce cyclic AMP levels in 1321N1 human astrocytoma cells. Our initial assumption that this effect occurred as a consequence of inhibition of adenylate cyclase was rejected on the basis of several lines of evidence.<sup>9</sup> Cholinergic stimuli decreased the time to

Department of Pharmacology and Program in Neurobiology, University of North Carolina School of Medicine, Chapel Hill, NC 27514, USA and Division of Pharmacology, University of California, San Diego, La Jolla, CA 92093, USA.

maximal cyclic AMP accumulation, an effect more consistent with an increase in cyclic AMP degradation; furthermore, no attenuation of cyclic AMP accumulation occurred in the presence of phosphodiesterase inhibitors. The fact that a marked increase in the rate of cyclic AMP degradation was caused by muscarinic receptor agonists whereas under no condition did muscarinic receptor agonists inhibit adenylate cyclase led us to conclude that activation of a cyclic AMP phosphodiesterase totally accounts for cholinergic effects on cyclic AMP accumulation in 1321N1 cells.

This idea was confirmed subsequently in studies using pertussis toxin.<sup>10</sup> Functional inactivation of  $G_i$  with toxin had no effect on muscarinic receptor-mediated attenuation of cyclic AMP accumulation in 1321N1 cells indicating that neither adenylate cyclase nor  $G_i$  were involved in cholinergic action in these cells. Subsequent work with selective inhibitors of phosphodiesterase has indicated that a  $Ca^{2+}$ -calmodulin regulated cyclic AMP phosphodiesterase is responsible for muscarinic receptor-mediated decreases in cyclic AMP levels in 1321N1 cells.

In light of this involvement of a  $Ca^{2+}$ -calmodulin regulated cyclic AMP phosphodiesterase, it was not surprising that cholinergic stimuli markedly increased the turnover of phosphoinositides in 1321N1 cells.<sup>11</sup> This effect, which could be measured as an increase in  $IP_3$ ,  $IP_2$ , and  $IP_1$  (Ref. 12), was accompanied by large increases in unidirectional efflux of  $^{45}Ca^{2+}$  from cells that had been prelabeled to steady state with  $^{45}CaCl_2$ . As had been demonstrated in a number of other tissues, the effect of cholinergic stimuli on phosphoinositide turnover was not secondary to an effect on the cytoplasmic  $Ca^{2+}$  concentration since the divalent cation ionophore A23187 had no effect on the level of inositol phosphates, and muscarinic receptor-mediated increases in metabolites of the polyphosphoinositides occurred normally in  $Ca^{2+}$ -free medium.

As discussed above, there is no evidence that muscarinic receptors of 1321N1 cells interact with  $G_i$ . Nevertheless, agonist interaction with muscarinic receptors in washed membrane preparations from these cells is characterized by complex binding curves that can be resolved into low and high affinity components.<sup>13</sup> Guanine nucleotides markedly shift these agonist competition curves to the right. Recently, we have demonstrated a positive correlation between the capacity of cholinergic agonists to induce the GTP-sensitive high affinity binding state (measured either as  $K_L/K_H$  or  $\%H$ ) and their efficacy for stimulation of  $Ca^{2+}$  mobilization or formation of inositol phosphates.<sup>14</sup> Pertussis toxin, which has been shown to inhibit coupling of inhibitory receptors to  $G_i$ , had no effect on agonist interaction with muscarinic receptors measured either in the absence or presence of GTP.<sup>13</sup> Thus, muscarinic receptors on 1321N1 cells apparently interact with a guanine nucleotide regulatory protein that is not  $G_i$ .

#### Muscarinic receptors of neuroblastoma x glioma cells

The results obtained with 1321N1 cells have been compared to those obtained with NG108-15 neuroblastoma x glioma cells. Muscarinic receptor-mediated inhibition of adenylate cyclase was readily observed in free preparations from NG108-15 cells. In addition, pertussis toxin pretreatment blocked the effect

of cholinergic stimuli on adenylate cyclase activity and completely prevented muscarinic receptor-mediated attenuation of cyclic AMP accumulation in intact cells.<sup>10</sup> These data, together with the fact that no evidence of muscarinic receptor-stimulated increases in phosphodiesterase activity has been obtained, have led us to conclude that inhibitory effects on adenylate cyclase completely account for cholinergic action in NG108-15 cells.

The binding properties of muscarinic receptor agonists in NG108-15 cells also differ from those in 1321N1 cells. As with 1321N1 cells, agonist competition curves with washed NG108-15 membranes could be computer-modeled into low and high affinity components, and guanine nucleotides steepened and shifted these curves to the right.<sup>13</sup> However, in contrast to 1321N1 cells, pertussis toxin pretreatment completely prevented the formation of a GTP-sensitive high affinity binding state for muscarinic receptor agonists.

#### Evidence for functionally distinct muscarinic receptors

One interpretation of the data obtained with the two cell lines is that the muscarinic receptors of 1321N1 cells are only capable of interacting with the phosphoinositide system, while those of NG108-15 cells only interact with  $G_i$  and adenylate cyclase. To make this conclusion, it was important to show that the adenylate cyclase system of

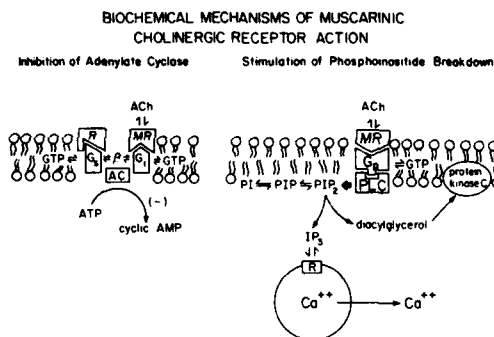


Fig. 1. Biochemical responses to stimulation of muscarinic cholinergic receptors. The proposed mechanism whereby acetylcholine (ACh) inhibits adenylate cyclase is presented on the left. The model indicates that ACh interacts with a muscarinic receptor (MR) to activate the inhibitory guanine nucleotide regulatory protein ( $G_i$ ). Inhibition of catalytic activity of adenylate cyclase (AC) then occurs probably by production of free  $\beta$ -subunit ( $\beta$ ) from  $G_i$  which 'deactivates' the activated  $\alpha$ -subunit of the stimulatory guanine nucleotide regulatory protein ( $G_s$ ). A stimulatory hormone receptor (R), e.g.  $\beta$ -adrenergic receptor, is also included as part of the model. The proposed mechanism whereby ACh stimulates phosphoinositide breakdown is presented on the right. ACh interacts with MR to activate a putative guanine nucleotide regulatory protein ( $G_p$ ), which in turn activates a membrane phospholipase C (PLC). Three phosphoinositides are depicted: phosphatidylinositol (PI), phosphatidyl inositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>). Activation of PLC results in hydrolysis of PIP<sub>2</sub> to the 'second messengers' inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol. IP<sub>3</sub> is depicted to interact with a site (•) on endoplasmic reticulum to release  $Ca^{2+}$ . Diacylglycerol activates protein kinase C.

1321N1 cells is fully responsive to an inhibitory receptor, e.g.  $G_i$  of 1321N1 cells is capable of functional interaction with both  $R_i$  and C, and likewise, that other hormones can stimulate the breakdown of phosphoinositides in NG108-15 cells. After screening a large number of possible inhibitory receptor agonists, it was discovered that adenosine receptor-mediated attenuation of cyclic AMP accumulation and inhibition of adenylate cyclase could be observed in 1321N1 cells. In contrast to the effects of muscarinic receptors on cyclic AMP accumulation, pertussis toxin completely blocked the adenosine receptor-mediated effects. Thus,  $G_i$  and adenylate cyclase of 1321N1 cells are apparently fully capable of responding to an inhibitory receptor. Yano *et al.*<sup>15</sup> have reported and we have confirmed (Hepler, J. R. and Harden, T. K., unpublished) that bradykinin markedly stimulates inositol phosphate formation in NG108-15 cells. Under the same conditions we have been unable to detect any effect of muscarinic receptor stimulation on either phosphoinositide breakdown or  $Ca^{2+}$  mobilization.

Taken together, the results with the two cell lines are consistent with the following proposals. A muscarinic receptor is expressed by 1321N1 cells that is incapable of coupling to  $G_i$  and adenylate cyclase. Nevertheless, this receptor does couple to an unidentified guanine nucleotide regulatory protein. The recent observation of guanine nucleotide-dependent stimulation of formation of inositol phosphates by carbachol in cell-free preparations from 1321N1 cells (Hepler, J. R., Nakahata, N. and Harden, T. K., unpublished observations) is consistent with the idea that this protein subserves a coupling function between muscarinic receptors and the phospholipase C involved in stimulation of phosphoinositide breakdown. We propose that the opposite situation is operative in NG108-15 cells. Here, a muscarinic receptor is expressed that interacts with  $G_i$  and adenylate cyclase but is incapable of functionally interfacing with the effector proteins responsible for the phosphoinositide response. Thus, two types of muscarinic receptors can be proposed on the basis of these studies with cultured cells: one receptor possesses a protein sequence that enables it to interact with  $G_i$  and, therefore, adenylate cyclase; the other receptor possesses a protein sequence that enables it to interact with a yet to be identified guanine nucleotide regulatory protein that interfaces receptors with phospholipase C.

Although we propose that the muscarinic receptors of the two cell lines can be distinguished based on their interaction with two different guanine nucleotide regulatory proteins and biochemical responses, they do not conform with the current terminology of  $M_1$ - and  $M_2$ -subtypes of muscarinic receptors that is based on the selectivity of the non-classical antagonist, pirenzepine (PZ).<sup>16</sup> If PZ indeed exhibits different affinities for different muscarinic receptor subtypes, then only one subtype is present on each cell line, since PZ competition curves with membranes from each cell line followed law of mass action interaction for a single site.<sup>17</sup> The affinity ( $K_i = 11$  nM) of PZ in competition binding experiments with 1321N1 membranes was similar to that proposed for the putative  $M_1$ -receptor subtype; however, the affinity ( $K_i = 25$  nM) of PZ for the muscarinic receptors of NG108-15 cells was only 2.5–3.0 fold lower. In addition, the  $K_i$  of PZ for antagonism of the phosphoinositide

response of 1321N1 cells<sup>18</sup> was closer to the low affinity value observed for PZ in competition binding experiments with various tissues.

#### Muscarinic receptors of chick heart cells

Muscarinic receptor-mediated inhibition of adenylate cyclase and stimulation of phosphoinositide hydrolysis both occur in embryonic chick heart cells. The receptors mediating these two responses were directly compared in studies using freshly dissociated cells from 13-day-old chick embryos.

Studies with a series of cholinergic agonists demonstrated several striking differences in the two responses. First, the concentration-effect curve for carbachol-stimulated phosphoinositide hydrolysis ( $EC_{50} = 20$   $\mu$ M) was to the right of that for inhibition by carbachol of cyclic AMP production ( $EC_{50} = 0.2$   $\mu$ M; Ref. 19). Second, although oxotremorine was as efficacious as carbachol in decreasing cyclic AMP accumulation, it caused little phosphoinositide hydrolysis relative to carbachol.<sup>19</sup>

There are two possible explanations for these data. First, carbachol and oxotremorine could have different affinities at the receptors that mediate the two responses. This does not appear to be the case, however, since agonist  $K_a$  values calculated after receptor alkylation are the same ( $\approx 40$   $\mu$ M) for either response. Instead there appear to be differences in the receptor reserve ('spare receptors') for these two responses. These conclusions come from studies in which responses were examined subsequent to inactivation of receptors by alkylation with propylbenzylcholine mustard (PBCM). Treatment of cells with  $10^{-8}$  M PBCM for 15 min alkylated more than 95% of muscarinic receptors as assessed by  $^3$ H-N-methylscopolamine binding. Under these conditions, the capacity of carbachol to stimulate phosphoinositide hydrolysis was virtually abolished, whereas carbachol could still fully inhibit cyclic AMP formation (Brown, J. H. *et al.*, unpublished observations).

The concentration-effect curve for inhibition of cyclic AMP formation by carbachol was markedly shifted to the right as receptors were alkylated, so that the  $K_i$  for carbachol increased from 0.2  $\mu$ M to 40  $\mu$ M after removal of receptor reserve. These data indicate that the difference in the two responses can be accounted for by a situation in which occupancy by carbachol of relatively few muscarinic receptors results in complete inhibition of adenylate cyclase while stimulation of phosphoinositide hydrolysis is related in a linear fashion to carbachol binding, i.e. maximal activation of phospholipase C in these cells requires full activation of all of the muscarinic receptors.

Similar arguments can explain the observation that oxotremorine and pilocarpine are effective agonists at muscarinic receptors regulating adenylate cyclase but are partial agonists relative to carbachol regarding stimulation of phosphoinositide hydrolysis in the chick heart<sup>19</sup> or in 1321N1 cells.<sup>11,14</sup> That is, partial activation of some or all of the muscarinic receptors is enough to fully inhibit adenylate cyclase; in contrast, the magnitude of the phosphoinositide response is limited because a maximal phosphoinositide response requires full activation of all of the available receptors. Data to date would suggest that the differences in properties of these two response systems lies at the level of receptor-effector coupling. Consistent

with this idea and the proposed role of a guanine nucleotide regulatory protein in the phosphoinositide response, the efficacy of agonists for stimulation of phosphoinositide hydrolysis in 1321N1 cells correlates with their capacity to induce formation of a high affinity GTP-sensitive binding state.<sup>14</sup>

Pirenzepine has been tested as an antagonist of the two cholinergic responses in the chick heart cell.  $K_i$  values were determined by Schild analysis of concentration-effect curves generated with different concentrations of PZ. Atropine blocked both responses with similar affinity ( $K_i = 1-2 \text{ nM}$ ). Pirenzepine showed some 'selectivity', blocking the cyclic AMP response with a  $K_i$  of 60 nM and the phosphoinositide response with a  $K_i$  of 240 nM (Ref. 18). If  $M_1$  and  $M_2$  receptors are response-specific, the direction of this small difference would lead one to conclude that the  $M_1$  receptor sites were more closely related to cyclic AMP responses than to phosphoinositide metabolism.

The agonist and antagonist data presented above suggest that the receptors that regulate the two biochemical responses to cholinergic stimuli do not differ substantially in their ligand binding properties but differ in the mechanism and efficiency with which they interact with the appropriate guanine nucleotide regulatory proteins. As was observed in our experiments with the cultured cell lines, it is at the level of the guanine nucleotide regulatory protein that the biochemical responses can be selectively blocked. Thus, in chick heart cells, pertussis toxin treatment resulted in a concentration-dependent blockade of muscarinic receptor-mediated inhibition of cyclic AMP formation; no effect on phosphoinositide hydrolysis was observed.<sup>19</sup> On the other hand, the phorbol ester 4 $\beta$ -phorbol 12 $\beta$ -myristate 13 $\alpha$ -acetate (PMA) antagonized muscarinic receptor-mediated phosphoinositide hydrolysis without blocking muscarinic receptor-mediated inhibition of cyclic AMP formation (Ref. 20, 21; Orellana, S. A. and Brown, J. H., unpublished observations). The site of action of PMA is not yet known but the fact that phosphoinositide hydrolysis stimulated through a number of different hormone receptors is blocked, suggests that PMA acts by modulating the function of the putative guanine nucleotide regulatory protein involved in coupling muscarinic receptors to the phospholipase C.

#### Selectivity of cholinergic action based on receptor-effector coupling

In conclusion, the data with 1321N1, NG108-15, and chick heart cells are consistent with the idea that the two major biochemical responses to cholinergic stimuli occur through muscarinic receptors that differ in their interaction with effector proteins in the plasma membrane. The response to cholinergic stimuli in NG108-15 cells and one of the responses in chick heart cells involves coupling of muscarinic receptors to  $G_i$  which subsequently results in an inhibition of adenylate cyclase. Based on agonist-binding data<sup>13,14</sup> and preliminary data indicating GTP-dependent formation of inositol phosphates in response to carbachol, we propose that the muscarinic receptors of 1321N1 cells couple to a guanine nucleotide regulatory protein ( $G_p$ ) that is not  $G_i$ . Receptor-mediated activation of this putative regulatory protein results in activation of a membrane phospholipase C. This suggestion is

supported by recent reports that demonstrate guanine nucleotide-dependent hydrolysis of phosphoinositides in other systems.<sup>22,23</sup>

Distinct polypeptide portions of receptor proteins must establish the selectivity of interaction of agonist-occupied receptors with effector proteins of the plasma membrane. Consequently, the data with NG108-15 and 1321N1 cells indicate that at least in these two cell lines, muscarinic receptors can effectively interact with either  $G_i$  or  $G_p$ , but not both. A deduction from these data then is that two muscarinic receptors can be defined based on their selectivity of interaction with guanine nucleotide regulatory proteins and the two second messenger systems. The properties of the two cholinergic responses in chick heart cells and the selective modification of these responses by perturbants that act at the level of a guanine nucleotide regulatory protein (pertussis toxin and PMA) support the conclusions derived from studies with the two cell lines. To date, our pharmacological data with these model cell systems have failed to support the proposal that the putative  $M_1$  muscarinic receptor couples to one of the biochemical responses while the putative  $M_2$  receptor mediates the other response. More data are clearly needed to establish if any pharmacological specificity is consistently expressed by receptors that exhibit selectivity in activation of one or the other of the second messenger systems.

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## Muscarinic receptors and membrane ion conductances

R. Alan North

*Muscarinic agonists appear to have four distinct actions on the membranes of excitable tissues. First, an increase in potassium conductance occurs in cardiac tissue, some gland cells and neurones. Second, a decrease in potassium conductance can be observed in a variety of neurones and in toad stomach smooth muscle. Third, an increase in cation (most importantly, sodium) conductance occurs in mammalian smooth muscle, some gland cells and neurones. Fourth, muscarinic agonists can directly reduce calcium conductance in cardiac muscle and neurones. Measurements of dissociation equilibrium constants for antagonists including pirenzepine show that the decrease in potassium conductance involves an  $M_1$  receptor, the other effects belong within the  $M_2$  receptor category but have not been distinguished from each other.*

A great variety of vertebrate cells show responses to acetylcholine (ACh) which can be mimicked by muscarine and blocked by atropine; these include endothelium, endocrine and exocrine glands, smooth muscles and several different types of neurones. In most of these cells, the occupancy of the receptor by the muscarinic agonist leads to a change in the ability of the cell membrane to allow the passage of ions down their electrochemical gradients, by opening or closing selective channels. There is evidence in one case that the receptor is in fact intimately associated with the ion channel<sup>1</sup>; in other cases it seems more likely that the alteration in ion conductance occurs as the result of changing levels of an intracellular or intramembrane intermediate.

The existence of several ion conductance changes in response to muscarinic agonists raises the possibility that the different conductance changes result from activation of distinct receptor molecules. This hypothesis is based on analogy with other receptor systems, in which distinct receptors are linked to particular changes in conductance.<sup>2</sup> The hypothesis is now being subjected to experimental tests, and the present paper reviews some of these experiments.

### Increase in potassium conductance

Muscarinic agonists increase membrane  $K^+$  conductance in heart,<sup>1,3-8</sup> autonomic ganglion cells,<sup>9,10</sup> gland cells,<sup>11-14</sup> and central neurones.<sup>15</sup> The muscarinic receptor subtype involved in this effect has been directly characterized in experiments on single neurones of the rat nucleus parabrachialis;<sup>15,16</sup> here the antagonism between muscarine and pirenzepine (PZ) is competitive and the PZ dissociation equilibrium constant ( $K_D$ ) is about 600 nM (within the class known as the  $M_2$  receptor) (Fig. 1). Other muscarinic increases in  $K^+$  conductance have not yet been studied with a view to determining the receptor subtype involved. However, there are two tissues in which less direct evidence is compatible with the notion that the receptors involved belong to the  $M_2$  class. First, cardiac slowing by acetylcholine (ACh), which presumably results from an increase in  $K^+$  conductance of pacemaker cells,

is antagonized by PZ with a  $K_D$  of approximately 600 nM.<sup>17</sup> Second, synaptic hyperpolarizations of intact rabbit superior cervical ganglia are blocked by gallamine, which is compatible with their being mediated by an  $M_2$  receptor.<sup>18</sup>

The properties of the  $K^+$  conductance which is increased by ACh have been studied in some detail. Nodal cells from the rabbit heart have  $K^+$  channels which are open at rest and which show marked inward rectification; the channels have a mean open time of about 1.4 ms and a unit conductance at the resting potential of about 35 pS (Ref. 1). Application of ACh to nodal cells causes channels to open which have the same properties. On the other hand, non-nodal cells from the atrium or ventricle had  $K^+$  channels of a similar unit conductance but much longer mean open time (about 50 ms); these cells did not show any channel openings in response to ACh (Refs 1, 7).

Acinar cells of pig pancreas provide another example of an increase in  $K^+$  conductance in response to ACh (and other secretagogues).<sup>12</sup> This channel is sensitive both to membrane voltage and to the intracellular calcium concentration, and is in a class of  $K^+$  channels having a large (about 200 pS) unit conductance. In the resting condition, with intracellular  $Ca^{2+}$  concentrations of 10–100 nM, there are about 50 such channels open in the membrane of a pig acinar cell.<sup>19</sup>

In the three sets of neurones so far examined in any detail, the muscarine activated  $K^+$  conductance is similar. It is essentially independent of membrane potential between about –40 and –110 mV (Refs 9, 10, 15); indicating that it may be different from that of the pig acinar cells. Single ACh activated  $K^+$  channels have not been studied in neurones; typical whole cell conductances are 5–10 nS. The conductance is remarkably similar in many of its properties to that which is increased by opioid receptor ( $\mu$  and  $\delta$ ) agonists and  $\alpha_2$ -adrenoceptor agonists in other neurones.<sup>2,20,21</sup>

The consequences to the cell of a muscarinic increase in  $K^+$  conductance can be profound, the details will vary according to the properties (e.g. voltage and/or  $Ca^{2+}$  dependence) of the individual channels. Gland cells hyperpolarize, but the main functional effect is thought to be the release of  $K^+$ . The potassium ions are probably then transported back into the cell by a  $K^+Na^+Cl^-$  co-

transport system; the cycling of potassium thus allows the movement of  $\text{Cl}^-$  in the direction of fluid secretion.<sup>12</sup> Neurones hyperpolarize; this can simply stop action potentials, or it can move the membrane to a potential at which different sets of conductances come into operation. Individual action potentials may be reduced in duration by the  $\text{K}^+$  conductance increase, and this could contribute to presynaptic inhibition of transmitter release (see below). Pacemaker cells, such as in the heart, slow down and bradycardia ensues. Different physiological consequences result from a common ion conductance change; it is clearly important to know both the detailed properties of the  $\text{K}^+$  conductance and the muscarinic receptor subtype which controls it in these various tissues.

#### Decrease in potassium conductance

The proposal that muscarinic agonists might decrease the  $\text{K}^+$  conductance of the cell membrane was first made for sympathetic ganglion cells<sup>22</sup> and cerebral cortical neurones.<sup>23</sup> Brown *et al.*<sup>24</sup> reported that the muscarinic depolarizations of rat sympathetic ganglion cells were blocked by relatively low concentrations of PZ; the depolarizations were recorded with extracellular electrodes, but most likely result from a closure of  $\text{K}^+$  channels. A direct demonstration of the receptor type involved in  $\text{K}^+$  inactivation has recently been made in enteric neurones.<sup>25</sup> In this case, the membrane depolarization was recorded with intracellular electrodes; it results from reduction in a 'resting'  $\text{K}^+$  conductance (see below). Antagonist dissociation equilibrium constants ( $K_D$ 's) were determined during the course of recordings from individual nerve cells; the PZ  $K_D$  of 5–10 nM indicates the involvement of an  $M_1$  receptor (Fig. 1).

The particular kind of potassium conductance that is decreased by muscarinic agonists has been the subject of some interest. It now appears that the properties of the affected conductance vary among different neurones, but in general the conductance can be assigned to one of three classes. These are a background conductance that contributes to the resting membrane potential, a  $\text{Ca}^{2+}$ -activated conductance that is responsible for a portion of the action potential afterhyperpolarization, and a voltage-sensitive conductance (the M-conductance) which is largely closed at resting membrane potentials but which opens within tens of milliseconds during depolarization. It should be emphasized that the appropriate experiments to distinguish among these types have not always been carried out, and that a single neurone can often show muscarinic effects on more than one of these conductance types. Indeed, it is possible that a single basic  $\text{K}^+$  channel is involved, and that its sensitivity to membrane potential and intracellular  $\text{Ca}^{2+}$  varies with the cell type and the experimental conditions.

Muscarinic inhibition of a resting  $\text{K}^+$  conductance has been most widely studied in the enteric neurones<sup>25, 28</sup> but also appears to play a role in hippocampal pyramidal cells.<sup>29</sup> The conductance affected seems to be essentially independent of membrane potential in the range of -50 to -120 mV; that is to say, the membrane channels are still open and sensitive to muscarinic agonists even at membrane potentials more negative than the  $\text{K}^+$  equilibrium potential ( $E_K$ ) (Ref. 26).

The actions of ACh on the afterhyperpolarization of the action potential were first described in enteric

neurones<sup>26, 27</sup> and have since been reported in bullfrog sympathetic ganglion cells<sup>29a</sup> and hippocampal pyramidal cells.<sup>30, 31</sup> There are some difficulties in analysing this action of muscarinic agonists, because the afterhyperpolarization results from  $\text{Ca}^{2+}$  entering the neurones during the action potential, and a depression of the afterhyperpolarization might result from a reduction in  $\text{Ca}^{2+}$  entry. This would represent a primary action of muscarinic agonists on the  $\text{Ca}^{2+}$  conductance (see below) rather than the  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  conductance. North and Tokimasa<sup>27</sup> excluded this possibility by applying the agonist (ACh) after the action potentials which carried the  $\text{Ca}^{2+}$  into the neurone. Furthermore, in bullfrog ganglion cells membrane hyperpolarizations can be observed after caffeine treatment that result from the spontaneous liberation of  $\text{Ca}^{2+}$  from intracellular stores; the suppression of these hyperpolarizations by ACh excludes a primary action on  $\text{Ca}^{2+}$  entry.<sup>32</sup> Suppression of the afterhyperpolarization by muscarinic agonists, particularly a reduction in its duration, will increase the rate at which the cell will discharge action potentials in response to other excitatory synaptic inputs.

Suppression of the M-current by muscarinic agonists has been observed in many neurones,<sup>33, 39</sup> as well as in isolated smooth muscle cells of the rod stomach.<sup>40</sup> The M-conductance opens as the membrane is depolarized from the resting level (around -60 mV); as the M-channels open, outward current flows through them, tending to hyperpolarize the membrane, and resulting in accommodation and cessation of action potential discharge. An important effect of M-current inhibition by muscarinic agonists is thus the continued firing of the cell in response to a depolarizing stimulus.

#### Increase in cation conductance

Muscarinic receptors on mammalian smooth muscle<sup>41, 42</sup> and some gland cells<sup>43, 45</sup> appear to be coupled to a conductance which is permeable to cations. This results in an inward current which depolarizes the cell. The receptor subtype responsible for these effects has not been directly characterized; however, the depolarization is an intermediate step in both contraction of the smooth muscle or secretion of the gland cells and these events are known to involve receptors within the  $M_3$  class. A somewhat similar action of muscarinic agonists has recently been described in neurones of the rat locus coeruleus.<sup>46</sup> In these cells, ACh causes an inward current which is carried largely by  $\text{Na}^+$ , and this depolarizes the cell and increases its rate of firing. Measurements of depolarization or firing rate were used to determine  $K_D$ 's for atropine and pirenzepine.<sup>47</sup> The PZ  $K_D$  was 230 nM, and the atropine  $K_D$  was less than 1 nM. The values for the PZ  $K_D$  on single neurones of rat locus coeruleus ( $\text{Na}^+$  conductance increase) show no overlap with those determined for single cells of the nearby nucleus parabrachialis ( $\text{K}^+$  conductance increase, see above), but a conclusive distinction between these receptor subtypes awaits investigation with antagonists of greater selectivity.

#### Decrease in calcium conductance

A depression of  $\text{Ca}^{2+}$  entry into bullfrog heart cells by ACh was described by Giles and Noble<sup>4</sup> and more recently for guinea-pig atria by Iijima *et al.*<sup>5</sup>. The direct

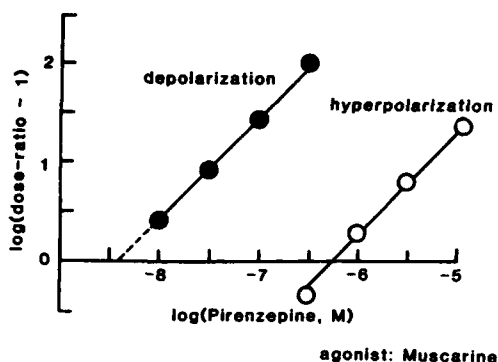


Fig. 1. Pirenzepine discriminates between the muscarinic receptors responsible for potassium conductance decrease and those responsible for potassium conductance increase. The graph shows the Schild plots for the antagonism of muscarine by pirenzepine. The left line (filled circles) was derived from an experiment on a single neurone of the guinea-pig submucous plexus, in which depolarizations by muscarine were recorded. The right line (open circles) was derived from an experiment on a single neurone of the rat nucleus parabrachialis, in which hyperpolarizations by muscarine were measured. It has been shown<sup>15,16,25,26</sup> that depolarizations and hyperpolarization result respectively from decreases or increases in the membrane potassium conductance.

action on inward  $\text{Ca}^{2+}$  current can be studied only after suppression of  $\text{K}^{+}$  currents, since the activation of a  $\text{K}^{+}$  current by ACh might not be distinguishable from a simultaneous depression of a  $\text{Ca}^{2+}$  current. Higher concentrations of ACh are required to inhibit the  $\text{Ca}^{2+}$  current than to increase  $\text{K}^{+}$  conductance.<sup>5</sup> It is unclear whether these two actions on the heart are mediated by the same or different receptors, and measurement of antagonist  $K_D$ s for each effect would be useful. There is a report<sup>17</sup> that the PZ  $K_D$  differs for measurements of the negative inotropic and negative chronotropic effects of muscarinic agonists, and it is possible that these two effects correspond at least in part to depression of  $\text{Ca}^{2+}$  conductance and increase in  $\text{K}^{+}$  conductance respectively. However, the situation is complicated because the amount of  $\text{Ca}^{2+}$  which enters the cell during the action potential and thereby influences the force of contraction, will depend not only on the  $\text{Ca}^{2+}$  conductance of the membrane, but on the duration of the action potential, which is very sensitive to the  $\text{K}^{+}$  conductance.

Muscarinic agonists inhibit the  $\text{Ca}^{2+}$  action potential in a variety of neurones (Refs 27, 48, 49 and Egan, T. M., Williams, J. T. and North, R. A., unpublished observations). A direct suppression of the  $\text{Ca}^{2+}$  current has been demonstrated in conditions in which effects on opposing outward currents have been suppressed (rat sympathetic ganglion cells<sup>48</sup> and locus coeruleus neurones (Egan, T. M. *et al.*, unpublished results)). The study of this direct suppression of  $\text{Ca}^{2+}$  conductance is at an early stage, and the underlying receptor has not been characterized by Schild analysis. The significance of this action of muscarinic agonists in nervous tissue may relate more to presynaptic inhibition than to the control of  $\text{Ca}^{2+}$  entry at the soma-dendritic membrane. The release of a variety of transmitters (ACh, noradrenaline, substance P) is

inhibited by muscarinic agonists,<sup>25,50,51</sup> and the receptor responsible is within the  $M_2$  class. Either an increase in  $\text{K}^{+}$  conductance or a reduction in  $\text{Ca}^{2+}$  conductance could contribute to presynaptic inhibition. If the receptor subtypes responsible for these two actions could be differentiated by recordings from the cell soma, in combination with  $K_D$  determinations, then it will be possible to compare the values with the  $K_D$ s for presynaptic inhibition of transmitter release from the same neurones.

#### Physiological significance

Whereas it is necessary to characterize the receptors involved by applying exogenous agonists and antagonists, the effects described above may also result from the actions of ACh released during physiological circumstances. The  $\text{K}^{+}$  conductance increase underlies synaptic hyperpolarizations in mudpuppy cardiac ganglion cells,<sup>10</sup> and in bullfrog C cells.<sup>9</sup> The  $\text{K}^{+}$  conductance decrease is responsible for one kind of slow e.p.s.p. in myenteric neurones,<sup>52</sup> and in bullfrog B cells.<sup>34</sup> Even the tiny amount of ACh which is released spontaneously to result in the miniature fast e.p.s.p.'s (which are nicotinic) can also activate muscarinic receptors. This was shown by Tokimasa,<sup>53</sup> who observed that the action potential afterhyperpolarization in bullfrog ganglion cells was shorter in duration when the action potential followed a few miniature e.p.s.p.'s than when it occurred without the miniature e.p.s.p.'s. ACh is one of the mediators of glandular secretion,<sup>11,12</sup> whether bringing about its effects by cation non-selective or by  $\text{K}^{+}$  conductance increase. It is not yet known whether a reduction of calcium entry might be a physiologically relevant action of ACh on muscarinic receptors, but there is no reason to believe that it is not.

#### Perspectives

The four actions of ACh described in this paper may each result from activation of a distinct receptor subtype; or, two or more of the actions may represent alternative responses of cells to activation of the same receptor subtype. The decrease in  $\text{K}^{+}$  conductance clearly involves the  $M_1$  receptor, whereas the other three actions can be ascribed to activation of  $M_2$  receptors. It is hypothesized that these three actions might also be distinguishable by determination of antagonist  $K_D$ s in conjunction with recordings from individual neurones; experimental tests of this hypothesis are awaited with new muscarinic antagonists.

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## Muscarinic receptors in the submucous plexus and their roles in mucosal ion transport

Annmarie Surprenant

*Recent immunohistochemical and ion transport studies have shown the neurones of the submucous plexus to be intimately involved in the control of intestinal ion transport. Muscarinic receptors which are present on both sides of the synapses in the submucous plexus were characterized by measuring changes in membrane potential (postsynaptic depolarization) and changes in the amplitude of nerve-evoked synaptic potentials (presynaptic inhibition of acetylcholine and noradrenaline release) during superfusion with known concentrations of muscarinic agonists and antagonists.  $pA_2$  values for pirenzepine showed that muscarinic depolarization of submucous neurones is due to activation of the  $M_1$  receptor subtype while inhibition of both acetylcholine and noradrenaline release is due to activation of the presynaptic  $M_2$  receptors. Possible functional roles for these muscarinic receptors in regulating secretory and absorptive activity of the intestinal mucosa are described.*

As early as the 1890's, cholinergic drugs were shown to increase water and electrolyte secretion in the intestine; further studies throughout most of the present century led to the concept that parasympathetic input directly onto the intestinal epithelia was responsible for the cholinergic stimulation of intestinal ion secretions.<sup>1,2</sup> It has also been known for over a century that millions of nerve cells lie within two distinct layers of the intestinal wall: the myenteric (Auerbach's) and the submucous (Meissner's) plexuses.<sup>3,4</sup> While it has long been recognized that myenteric neurones mediate intrinsic gastrointestinal reflexes (e.g. peristalsis) which underlie the co-ordinated movements of the intestinal smooth muscle, it is only in the last few years that convincing evidence has begun to accumulate which suggests submucous neurones may play an analogous role in controlling intestinal ion transport.

Recent quantitative immunohistochemical mapping of neurones in the submucous plexus of the small intestine has demonstrated that virtually all fibres emanating from submucous neurones project either to other submucous neurones or into the intestinal mucosa.<sup>5-9</sup> Of particular significance, in terms of muscarinic effects, has been the demonstration that about 50% of submucous neurones in the guinea-pig ileum contain choline acetyltransferase,<sup>9</sup> which implies that half of all submucous plexus neurones are cholinergic. Neuronal cell bodies in the submucous plexus also contain most of the known neuropeptides (with the notable exception of the enkephalins), in particular vasoactive intestinal polypeptide (VIP) which, like acetylcholine (ACh), exerts a marked secretory response in intestinal epithelia.<sup>5-10</sup> Adrenergic innervation of submucous plexus neurones and intestinal mucosa provides the primary source of neurally-mediated absorptive activity.<sup>1</sup> The only source of adrenergic innervation to the gastrointestinal epithelia is extrinsic, via sympathetic axons which come into the submucous plexus with the mesenteric blood vessels.<sup>8</sup> Some sympathetic axons pass directly through the submucous plexus to end in the mucosal crypts; however, a very large number also end on submucous

neurones themselves. Moreover, ion transport studies *in vitro*, using the Ussing chamber technique, have shown that secretion or absorption in response to cholinergic agonists, adrenergic agonists, or peptides, require the functional integrity of the submucous plexus.<sup>1,2,10-13</sup>

The functional properties of nerve cells in the submucous plexus of the guinea-pig small intestine and caecum have been studied using intracellular recording techniques.<sup>14-21</sup> These electrophysiological studies have shown that the majority of submucous plexus neurones display uniform membrane properties,<sup>14,16,20</sup> with the exception of a small group (about 5%, the AH cells<sup>21</sup>).

### Muscarinic receptors on cell bodies of submucous neurones

The muscarinic receptor which is present on the cell body of a single submucous neurone can be characterized in a very straightforward manner: a microelectrode is inserted into the cell soma, the change in membrane potential (or membrane current recorded under voltage-clamp conditions) is recorded during superfusion with several known concentrations of agonists; this is repeated in the presence of three to four antagonist concentrations and the resulting dose-response curves are analysed by the method of Arunlakshana and Schild.<sup>22</sup> In the guinea-pig small intestine, we found that muscarinic agonists depolarized approximately 30% of submucous plexus neurones; muscarinic agonists (up to 0.5 mM) caused no change in membrane potential in the remaining neurones. This depolarization was dose-dependent and the rank order of potency of the agonists we examined was oxotremorine > McN-A-343 = muscarine > bethanechol (Fig. 1). The muscarinic depolarization in submucous neurones was found to be due to an inward current which resulted from a closure of  $K^+$  channels in the membrane, as has been described previously for the actions of muscarinic agonists on myenteric plexus neurones.<sup>23</sup> Low concentrations of atropine, 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) and pirenzepine (PZ) antagonized the effects of muscarinic agonists. Schild plots obtained for this antagonism were straight with unit slopes and the PZ  $pA_2$  value was 8.5 (Fig. 2, Table I); thus, muscarinic depolarization of submucous neurones results from activation of postsynaptic  $M_1$  receptors.

Gastroenterology Division, Department of Medicine, Beth Israel Hospital, Harvard Medical School, 330 Brookline Avenue, Boston, MA 02215, USA.

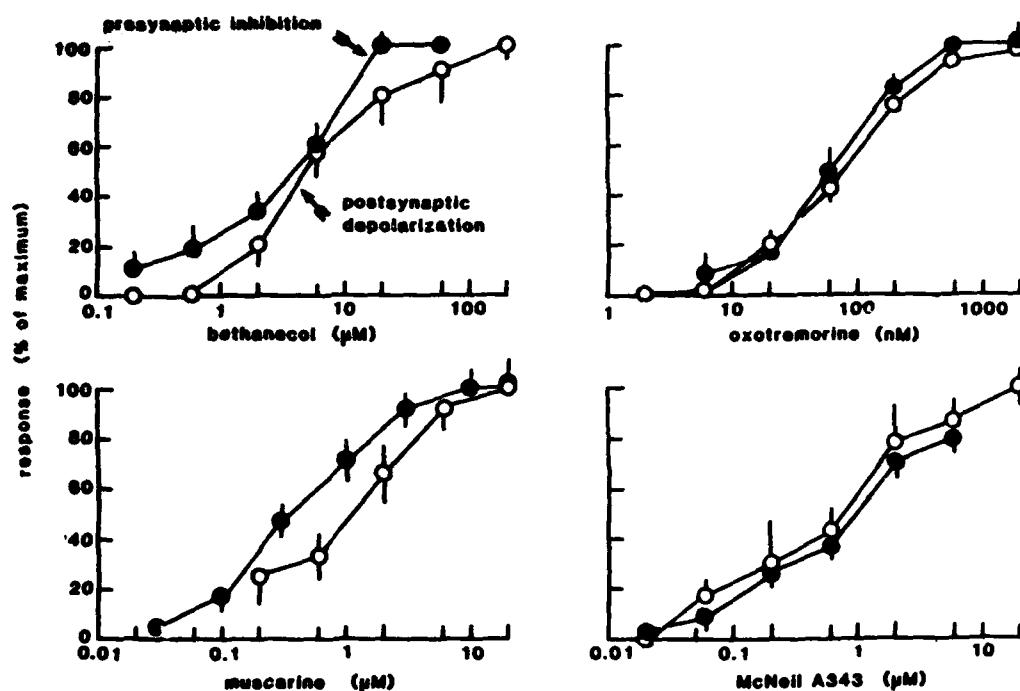


Fig. 1. Pre- and post-synaptic effects of muscarinic agonists on submucous plexus neurones of the guinea-pig ileum. Agonist-induced membrane depolarizations (○) are expressed as % of maximum depolarization recorded from individual neurones; each point represents the mean  $\pm$  S.E. of mean,  $n > 4$  for each point. Maximum depolarizations were 16–30 mV from resting membrane potentials of  $-48$  mV to  $-68$  mV in all submucous neurones which were depolarized by the application of these muscarinic agonists (approximately 30% of all neurones examined). Agonist-induced depression of the nicotinic fast e.p.s.p. (●), which represents presynaptic inhibition of ACh release, is expressed as % of maximum depression;  $n > 8$  for all points. These values were obtained from those submucous neurones which were not depolarized by the application of muscarinic agonists.

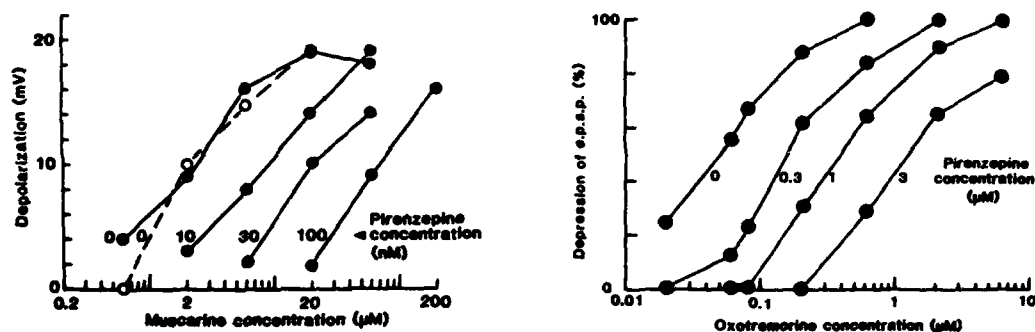


Fig. 2. A. Muscarine-induced depolarization recorded from one submucous neurone in the absence and then presence of increasing concentrations (10, 30 and 100 nM) of pirenzepine. These low doses of pirenzepine shifted the curves to the right in a parallel and competitive manner. Approximately 35 min after washing out the antagonist, depolarizations in response to muscarine had returned to control values (open circles). Schild plot of these data yielded a  $pA_2$  value of 8.4 (slope of 1.06). B. Oxotremorine-induced depression of the fast e.p.s.p. in the absence and presence of increasing concentrations (0.3, 1 and 3  $\mu$ M) of pirenzepine. These higher concentrations of pirenzepine produced a parallel, dose-dependent shift to the right; values obtained whilst recording from a single neurone. Results are expressed as % depression from control e.p.s.p. amplitude. Schild plot of these data gave a  $pA_2$  value of 6.8 (slope of 1.06).

**Table I.** Antagonist  $pA_2$  values obtained in submucous plexus neurones for pre- and post-synaptic effects of muscarinic agonists. Agonists were muscarine, oxotremorine, McN-A-343, bethanechol, or methylfurmethide. Values are expressed as mean  $\pm$  S.E. of mean where  $n$  (numbers in parentheses) is greater than two.

	Postsynaptic depolarization		fast e.p.s.p.		Presynaptic inhibition i.p.s.p.		slow e.p.s.p.	
	$pA_2$	slope	$pA_2$	slope	$pA_2$	slope	$pA_2$	slope
Pirenzepine	8.4;8.5	1.06;1.1	$7.0 \pm 0.04$	$1.1 \pm 0.16$ (13)	$6.9 \pm 0.1$	$1.02 \pm 0.15$ (5)	$7.1 \pm 0.3$	$1.1 \pm 0.2$ (4)
4-DAMP	8.5;8.9	0.96;1.1	$8.7 \pm 0.1$	$1.0 \pm 0.08$ (7)	$8.5 \pm 0.2$	$0.98 \pm 0.2$ (4)	8.9;8.4	0.95;1.12
Atropine	9.0	1.12	9.0;8.9	1.06;0.95	8.9	1.08	—	—

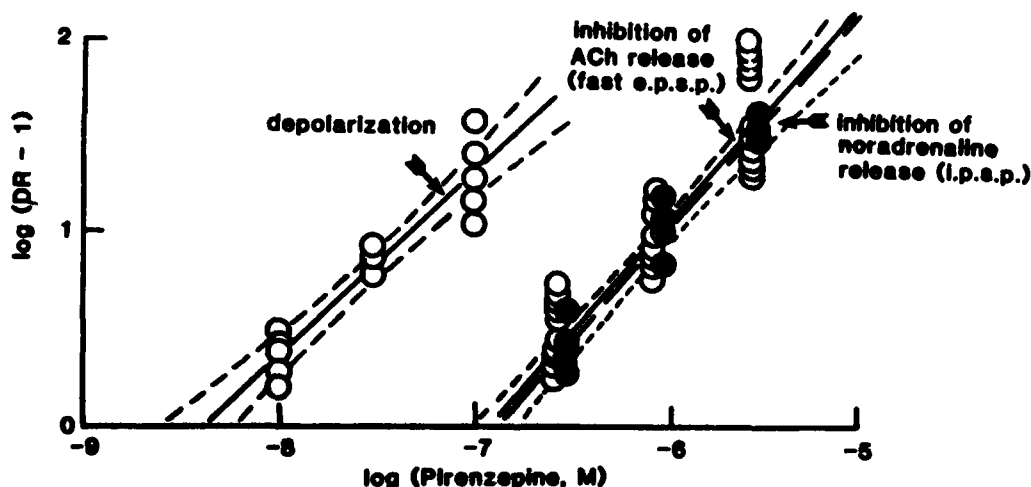
#### Characterization of presynaptic muscarinic receptors in the submucous plexus

It is also possible to characterize the muscarinic receptors which are present on nerve terminals of the cholinergic, adrenergic and peptide-containing fibres which project onto the submucous neuronal cell bodies because the amount of transmitter released from the nerve terminals in response to electrical stimulation of their nerve fibres can be assayed by recording discrete synaptic potentials in the cell body.

**ACh release.** When ACh is released from cholinergic nerve terminals, it activates nicotinic receptors which are present on submucous neurones and produces a brief (20–60 ms long) depolarization, the fast excitatory post-synaptic potential (fast e.p.s.p.).<sup>14</sup> The fast e.p.s.p. is due to a short-lasting (a few ms) increase in conductance to both  $Na^+$  and  $K^+$ , resulting in a net inward membrane current.<sup>23</sup> Muscarinic agonists produced a concentration-dependent depression in the amplitude of the fast e.p.s.p. in all submucous neurones (Fig. 1); this effect was due to a decreased amount of ACh released per nerve impulse because the postsynaptic nicotinic response to iono-

phoretic application of ACh (which mimicks the fast e.p.s.p.) was unaltered in the presence of muscarinic agonists. The rank order of potency of muscarinic agonists for the depression of the fast e.p.s.p. was oxotremorine > muscarine = methylfurmethide = McN-A-343 > bethanechol (Fig. 1); i.e. similar to that found for the postsynaptic muscarinic depolarization. Atropine, 4-DAMP, and PZ alone, had no effect on the amplitude of the fast e.p.s.p. evoked in response to single or low frequency (less than 2 Hz) stimulation but they all prevented the muscarinic depression of the fast e.p.s.p. Pirenzepine (greater than 100 nM) shifted the concentration-response curves to the right, in a parallel and competitive fashion (Fig. 2). The PZ  $pA_2$  value for presynaptic depression of ACh release was approximately 7 (Fig. 3; Table I).

**Noradrenaline release.** Release of noradrenaline (NA) from sympathetic nerve fibres projecting onto submucous neurones activates postsynaptic  $\alpha_2$ -adrenoceptors; this opens  $K^+$  channels in the membrane and results in a hyperpolarization of 1–2 s duration, the inhibitory post-synaptic potential (i.p.s.p.).<sup>18</sup> The amplitude of the i.p.s.p.



**Fig. 3.** Summary of results obtained with pirenzepine. Schild plots for the antagonism of muscarinic agonist-induced membrane depolarization, inhibition of the fast e.p.s.p. and inhibition of the i.p.s.p. in submucous neurones. Dashed lines are the 95% confidence limits of the linear regression line.

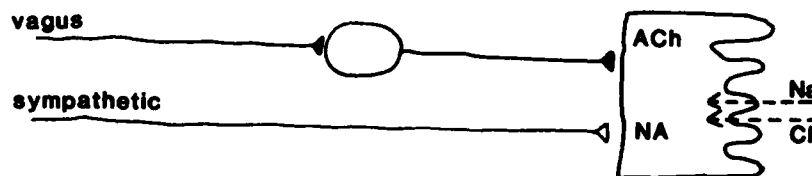
evoked by two or three shocks to the presynaptic nerve fibres is 25–30 mV (Refs 16, 18), this amplitude is close to the maximum possible because it represents the approach of the membrane potential to the  $K^+$  equilibrium potential ( $E_K$ ); therefore this sympathetic input provides a very powerful means of inhibiting neural activity in the submucous plexus.

Muscarinic agonists depressed the amplitude of the i.p.s.p. in a concentration-dependent manner; the order of potency was similar to that observed for the muscarinic depolarization as well as for the muscarinic depression of the fast e.p.s.p. Muscarinic antagonists prevented the inhibition of the i.p.s.p., and concentration-response curves obtained on individual neurones revealed parallel and competitive shifts to the right by 4-DAMP, atropine and PZ. Schild plots for the antagonism of the muscarinic depression of the i.p.s.p. by PZ were straight lines with

unit slope;  $pA_{50}$  values were approximately 7 (Fig. 3; Table I).

Atropine, 4-DAMP or PZ, alone, increased the amplitude and duration of the i.p.s.p. recorded in response to single, as well as multiple, nerve impulses. This increase in the i.p.s.p. amplitude was observed with low concentrations of these muscarinic antagonists [and did not become greater with increasing concentrations of the antagonists.] The enhancement of the i.p.s.p. by muscarinic antagonists appeared not to be due to any postsynaptic action of these drugs because the membrane hyperpolarization evoked by pressure or ionophoretic application of noradrenaline was not changed by the muscarinic antagonists. These results suggest that noradrenaline release in the submucous plexus is inhibited by endogenous ACh. The finding that the single-shock i.p.s.p. was also increased by the antagonists implies that

### classical control



### submucous control

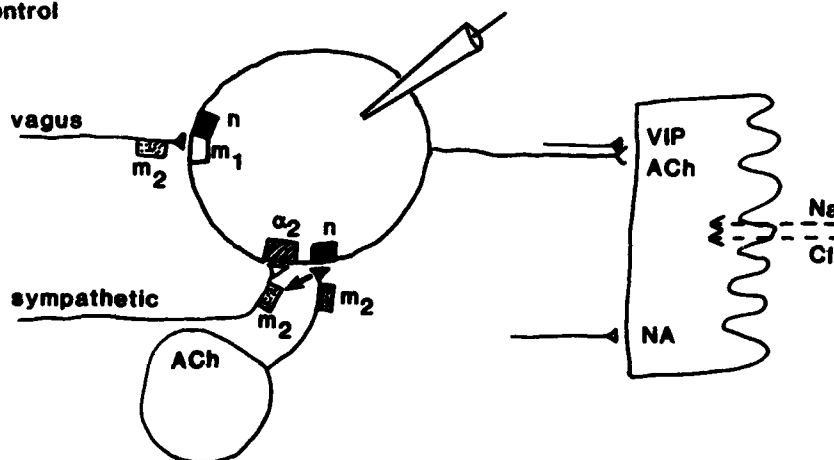


Fig. 4. Schematic of proposed roles for muscarinic receptors in controlling mucosal transport of electrolytes. Upper diagram shows classical control system: sympathetic fibres synapse directly onto mucosal cells where the release of NA causes absorption of electrolytes and water; vagal fibres synapse onto postganglionic cholinergic neurones which release ACh onto mucosal cells thereby exerting secretory actions. Lower diagram shows neural control of ion transport via submucous plexus neurones: ongoing release of NA from sympathetic fibres onto submucous neurones tonically inhibits release of ACh and neuropeptides;  $M_2$  receptors on sympathetic terminals, which are also tonically active, modulate this dominating sympathetic drive. Release of ACh from vagal fibres or cholinergic neurones in the submucous plexus can activate postsynaptic  $M_1$  (and nicotinic) receptors present on ACh- and peptide-containing neurones; this can lead to net electrolyte secretion by increasing the release of ACh and excitatory neuropeptides onto mucosal epithelia. Presynaptic  $M_2$  receptors on cholinergic and peptide-containing neurones probably come into play during excessive stimulation of submucous neurones where they can act to brake runaway secretory activity.

sufficient endogenous ACh is present to produce an ongoing occupancy and tonic inhibition of noradrenaline release.

**Neuropeptide release.** In addition to nicotinic fast e.p.s.p.'s and adrenergic i.p.s.p.'s, single and multiple stimuli applied to the interganglionic fibres tracts in the submucous plexus evokes a very slow (5 to 60 s long) depolarization in submucous neurones, the slow e.p.s.p.<sup>16,20</sup>. The slow e.p.s.p. is caused by closing  $K^+$  channels in the membrane and can be mimicked by the application of a number of neuropeptides (particularly substance P and VIP) which are known to be present in nerve fibres of the submucous plexus; it can also be mimicked by activation of the postsynaptic muscarinic receptors by ACh.<sup>16,19</sup> Since all of these substances are released during nerve stimulation, it currently seems most likely that the slow e.p.s.p. recorded from submucous neurones is the result of the simultaneous release of these neurotransmitters, each of which activates separate populations of receptors, all of which act to close the same population of  $K^+$  channels.

Muscarinic agonists depressed the amplitude of the slow e.p.s.p. in all submucous neurones. Pirenzepine, 4-DAMP and atropine, alone, had no effect on the amplitude or duration of the slow e.p.s.p.; but they competitively antagonized the depression by muscarinic agonists. Pirenzepine  $pA_2$  values demonstrated the existence of  $M_2$  receptors on the nerve terminals whose transmitters give rise to the slow e.p.s.p. (Table I).

#### Functional roles for muscarinic receptors in the regulation of intestinal ion transport

The schematic presented in Figure 4 illustrates the locations of, and proposed physiological roles for muscarinic receptors in the submucous plexus. Under physiological conditions in the body, there is net intestinal absorption of  $Na^+$  and  $Cl^-$ ; this is due primarily to the existence of a dominant sympathetic tone.<sup>1</sup> Thus, it is likely that ongoing adrenergic inhibition of cholinergic and peptide-containing neurones in the submucous plexus predominates in the 'basal' state. This adrenergic inhibition can be fine-tuned by small changes in the amounts of ACh which is producing an ongoing activation of presynaptic inhibition through  $M_2$  receptors on the sympathetic nerve terminals. The presence of these tonically active  $M_2$  receptors may serve to prevent excessive reabsorption which would result from a relative surfeit of noradrenaline release.

Studies of myenteric plexus preparations have shown that, at high frequencies of stimulation, endogenously released ACh can inhibit its own further release.<sup>24-27</sup> If a similar situation exists in the submucous plexus, it is likely that presynaptic  $M_2$  receptors on cholinergic and peptide-containing nerve fibres (neurones that primarily mediate increased secretory activity in the intestinal mucosa) may be activated during pathological states which result in high levels of cholinergic and/or peptidergic action potential activity; their presence then would serve to brake runaway diarrheal conditions.

On the other hand, increased cholinergic activity, either through the vagus, or due to various intraluminal stimuli in the form of nutritional, mechanical or osmotic variations, which may stimulate cholinergic neurones as

part of local reflex pathways, will activate postsynaptic  $M_1$  receptors on submucous neurones.  $M_1$  receptor activation will depolarize submucous neurones and thus directly increase  $Na^+$ ,  $Cl^-$  and water secretion in the intestinal mucosa by the release of ACh and other excitatory neuropeptides onto the mucosal epithelia. Additionally, the increased release of ACh due to activation of the postsynaptic  $M_1$  receptors on cholinergic neurones in the submucous plexus will further shut down noradrenaline release by activating the presynaptic sympathetic  $M_2$  receptors, thus enhancing the secretory processes by this submucosal modification of the traditional autonomic push-pull relationship.

#### Acknowledgement

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## A mechanism of muscarinic excitation in dissociated smooth muscle cells

Stephen M. Sims, Joshua J. Singer and John V. Walsh, Jr

*Dissociated gastric smooth muscle cells exhibit a potassium current resembling the M-current of sympathetic ganglia and other neurons, which is suppressed by muscarinic agonists. Suppression of this  $K^+$  current causes depolarization, which in turn leads to activation of voltage-sensitive  $Ca^{2+}$  channels and then contraction of the cell. The existence of a cholinergic  $K^+$  conductance decrease in smooth muscle is of interest because it provides an exception to the widely held view that muscarinic depolarization of smooth muscle is mediated exclusively by a membrane conductance increase for  $Na^+$ ,  $Ca^{2+}$  and  $K^+$  ions.*

Acetylcholine (ACh), acting on muscarinic receptors, is the major excitatory neurotransmitter in visceral smooth muscle of vertebrates, causing depolarization and contraction. For mammalian visceral smooth muscle the prevailing view has long been that muscarinic agonists directly cause a conductance increase for several ions, such as  $Na^+$ ,  $Ca^{2+}$  and  $K^+$  (Refs 1, 2).

In neural cells, the effector mechanisms underlying muscarinic excitation have been extensively characterized. Acetylcholine and other muscarinic agonists cause a depolarization as a result of a decrease in the permeability of the membrane for  $K^+$  ions.<sup>3-6</sup> The reduction of outward  $K^+$  current results in a net inward current across the membrane. This net inward current causes depolarization, which in turn can activate other voltage-sensitive conductances. Studies have revealed at least two distinct  $K^+$  currents that are suppressed by cholinergic agonists: these include the M-current<sup>7-15</sup> and a  $Ca^{2+}$ -activated  $K^+$  current.<sup>16-19</sup>

### Studies in single, freshly dissociated smooth muscle cells

We have chosen to examine muscarinic effector mechanisms in smooth muscle by using single smooth muscle cells obtained by enzymatic dissociation of the stomach of the toad.<sup>20-22</sup> Such a dispersed cell preparation eliminates many of the problems associated with studies of neurotransmitter actions on intact tissue preparations.<sup>23,24</sup> As an example, in tissue, neural elements may lead to indirect effects on the smooth muscle membrane when transmitters are applied or ionic conditions altered.

The disaggregated smooth muscle cells examined here have been extensively studied and shown to be viable by a wide variety of criteria. Their electrical properties have been characterized using current-clamp,<sup>23,25</sup> voltage-clamp<sup>26</sup> and patch-clamp techniques.<sup>27</sup> They have been shown to contract in response to ACh and carbachol (Ref. 28 and see below), and the apparent dissociation constant for antagonism by atropine of the ACh response was shown to be of the same order of magnitude as in the intact tissue.<sup>29</sup> In addition the cells respond to several peptides<sup>22</sup> and relax in response to adrenergic agents.<sup>30</sup> Therefore, the cells appear to emerge from the dis-

sociation procedure with a wide variety of receptors and functions intact.

### Muscarinic agonists cause excitation of single smooth muscle cells

The excitatory effects of cholinergic agonists on toad stomach have been characterized in the whole organ,<sup>31</sup> in intact muscle strips and in the dissociated cells.<sup>28,29</sup> Fig. 1 illustrates an ACh-induced contraction of a single smooth muscle cell. Prior to application of ACh, the cell was long and relaxed. Shortly after application of ACh the cell contracted to approximately 35% of its initial length, followed several minutes later by relaxation.

The question may be asked, how do muscarinic agonists cause contraction of smooth muscle cells? Intracellular microelectrode recording techniques have been used to investigate this problem. As shown in Fig. 1B, muscarine caused depolarization of the cell membrane, which initiated a burst of action potentials, the rising phase of which is due to  $Ca^{2+}$  current.<sup>25,26</sup> The subsequent elevation of cytoplasmic  $Ca^{2+}$  concentration led to contraction of this cell.

It is difficult to determine the primary effect of muscarine in experiments of the sort illustrated in Fig. 1B because the initial depolarization caused by muscarine can induce secondary changes, such as activation of voltage-sensitive conductances. In order to eliminate any contribution of the depolarization itself to the analysis of neurotransmitter actions, it is necessary to maintain the membrane potential constant (i.e. by using the voltage-clamp technique), as described in the following sections.

### Acetylcholine suppresses a $K^+$ current

In order to examine the conductance mechanisms affected by ACh, a single microelectrode voltage-clamp technique was employed (see details in Ref. 32). The use of conventional microelectrodes offered the advantage of minimal disturbance of the cytoplasm in comparison to the dialysis of the cell which occurs when patch electrodes are used for whole-cell recording.<sup>33</sup> The results of applying muscarine to a cell held at different membrane potentials are illustrated in Fig. 2A. The current induced by muscarine was inward at depolarized levels, and reversed direction to outward current when the potential was held more negative (panel A). The potential at which the agonist-induced current reversed direction coincided

with the predicted equilibrium potential for potassium ( $E_K$ , panel B). These observations indicate that muscarinic agonists modulate a  $K^+$  current, causing an inward current at potentials positive to  $E_K$  (corresponding to a depolarizing current in an unclamped cell) and an outward current at potentials negative to  $E_K$ . Therefore, cholinergic agonists must turn off a  $K^+$  current, that is, close channels permeable mainly to  $K^+$  ions. The close correspondence between the reversal potential and the predicted value of  $E_K$  over a range of potentials (Fig. 2B) indicates that muscarine has little, if any, effect on channels other than  $K^+$  channels under these conditions.

#### Voltage-clamp studies reveal M-current in smooth muscle cells

Further insight into the mechanism of ACh action was obtained by using the voltage-clamp protocol illustrated in Fig. 3. The membrane potential was periodically hyperpolarized from a  $-25$  mV holding level to  $-60$  mV. In the absence of ACh, the hyperpolarizing voltage command resulted in an initial rapid current jump followed by a slow change, or relaxation, to a steady-state level. As we described in Ref. 32, this current relaxation represents the turning off of an outward  $K^+$  current at more negative potentials. At the end of 2 s, when the membrane potential was returned to  $-25$  mV, there was another rapid current jump (though it is smaller in size because the  $K^+$  current had turned off at the more

negative potential) followed by the slow development of an outward current. This outward current relaxation represents the same  $K^+$  current turning back on at more positive potentials. Thus, a voltage-dependent  $K^+$  current is revealed with this voltage-clamp protocol, turning on at depolarized levels and turning off at more negative potentials.

Application of ACh to this cell caused the development of a net inward (depolarizing) current at  $-25$  mV, just as occurred in Fig. 2A. However, as shown in the middle expanded trace of Fig. 3, after ACh application, the current relaxations (reflecting the voltage-sensitive  $K^+$  conductance) have been abolished. Furthermore, the rapid current jump seen at the onset of the voltage jump was smaller. These results demonstrate that muscarinic agonists cause a  $K^+$  conductance decrease, and that the same  $K^+$  current that is suppressed by muscarinic agonists in these cells is also voltage-dependent. The features of the current described here (and described in detail in Ref. 32) resemble those of the M-current that was first identified in sympathetic ganglion neurons.<sup>7,10,11</sup>

#### Nature of the acetylcholine receptor

There are a number of indications that the cholinergic responses described here are mediated by muscarinic receptors. First, contractions induced by cholinergic agonists are antagonized by atropine.<sup>29</sup> Second, the suppression of M-current by cholinergic agonists (illus-

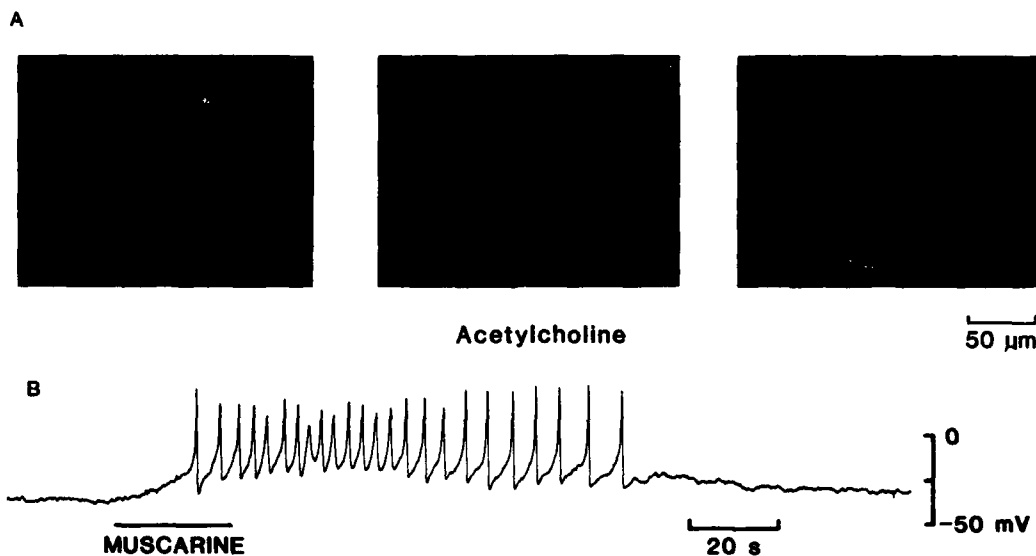


Fig. 1. Cholinergic agonists cause excitation and contraction of single gastric smooth muscle cells. A. The contraction of an isolated smooth muscle cell in response to acetylcholine (ACh) is shown in this series of video images. ACh was applied by pneumatic pressure ejection from a pipette ( $50 \mu$ M ACh in the application pipette), the tip of which can be seen at the lower right of each frame. The same cell is shown just prior to application of ACh, 15 seconds after onset of ACh application and 4 minutes later, after recovery. B. Recording of membrane potential illustrates an excitatory response of another cell to muscarine, applied for the time indicated by the bar below the voltage trace. The methods have been described in detail in Ref. 32. Muscarine ( $100 \mu$ M in the application pipette) caused depolarization of the membrane, which, at a threshold level, caused initiation of a burst of action potentials. Inward  $Ca^{2+}$  current is responsible for the rising phase of the action potentials<sup>25,26</sup> which in turn resulted in contraction of the cell. The cell relaxed during the quiescent recovery period shown at right. Electrical recordings of this sort illustrate the membrane events corresponding to the contraction shown in Panel A.

trated above) is also antagonized by atropine. (Measurements of membrane currents are worthwhile, because they offer a more direct index of receptor activation than does contraction.) Finally, the muscarinic agonists muscarine and oxotremorine mimic ACh in suppression of M-current.<sup>32</sup>

A wealth of evidence indicates that muscarinic receptors are not of a uniform type in all tissues. One distinction is that the receptors in smooth muscle differ from those found in some other tissues, such as some central and sympathetic neurons.<sup>4-36</sup> By one convention, muscarinic receptors mediating excitation in sympathetic neurons, for example, are termed  $M_1$ , while the receptors on gastrointestinal smooth muscle are designated  $M_2$ .<sup>39,40</sup>

Different receptor subtypes have been associated with apparent differences in effector mechanisms. Muscarinic depolarization in sympathetic neurons appears to be mediated by  $M_1$  type receptors,<sup>34</sup> and that depolarization has, in separate studies, been associated with a  $K^+$  conductance decrease (see Ref. 8 and also 7, 10, 11, 41). The second subtype of receptor ( $M_2$ ) has been identified in smooth muscle tissues<sup>34-36</sup> and, again from separate studies, the mechanism thought to underlie muscarinic depolarization leading to contraction in smooth muscle has been a conductance increase for several ions.<sup>12</sup> Our results show that a muscarinic  $K^+$  conductance decrease exists in at least some smooth muscle, a tissue presumed to have  $M_2$  receptors.

The question arises, what receptor subtype is involved in regulation of the  $K^+$  conductance (M-current) in toad gastric smooth muscle cells? McN-A-343, which is thought to selectively activate  $M_1$  receptors, was without effect on these cells in doses as high as 0.5 mM in the application pipette (unpublished observations). However, species differences may account for such a negative

finding, since McN-A-343 had no effect on amphibian sympathetic ganglion cells.<sup>42</sup> Therefore, lack of effect of McN-A-343 is inconclusive.

Pirenzepine (PZ) offers a more promising approach to identifying the receptor subtype coupled to the M-current in smooth muscle. Pirenzepine discriminates between different receptor types, binding tightly and acting as a relatively selective  $M_1$  antagonist,<sup>36,40</sup> while atropine shows no such selectivity, binding to  $M_1$  and  $M_2$  subtypes alike.

In preliminary studies we have compared the efficacy with which atropine and PZ block the response of M-current in smooth muscle cells to a standard dose of ACh (unpublished observations). In the presence of atropine (1  $\mu$ M), ACh had virtually no effect on M-current (see also Ref. 32). With PZ (1 and 10  $\mu$ M) present, however, ACh did still substantially inhibit the M-current. The lesser sensitivity of the receptors to PZ supports the interpretation that the receptors mediating suppression of M-current in smooth muscle cells are not of the  $M_1$  type. This interpretation is preliminary. We cannot yet rule out other factors, such as species differences, as contributing to the lack of effect of PZ on these cells. Further studies are required, for example, to determine if still higher doses of PZ are capable of antagonizing the actions of ACh.

#### Other agents can affect M-current

Studies of bullfrog sympathetic ganglion neurons have revealed that several peptides, such as substance P and luteinizing-hormone releasing-hormone, exert their excitatory influences by suppressing the M-current.<sup>11,43,44</sup> The convergence of different receptors to a common effector mechanism is also evident in toad gastric smooth muscle cells. Both ACh and substance P cause contraction of

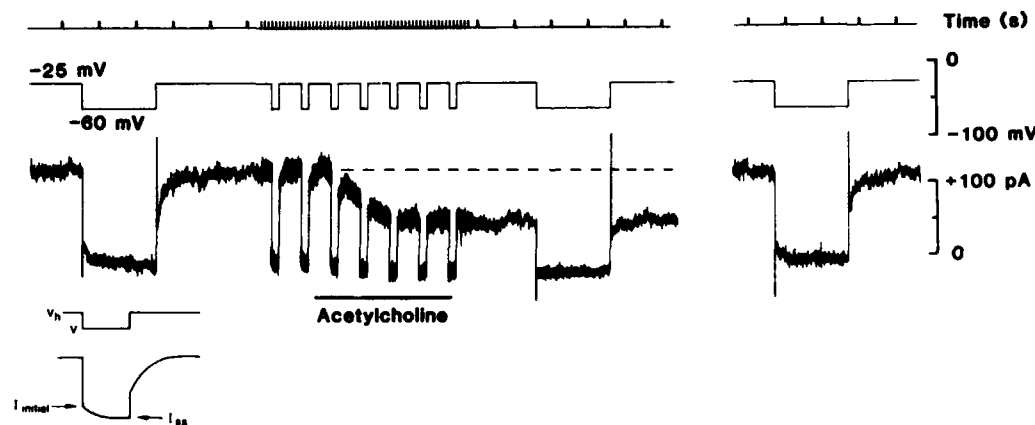


Fig. 2. Reversal of muscarine-induced currents is dependent upon the external  $K^+$  concentration ( $[K^+]_{out}$ ). A. Current records showing the responses to brief applications of muscarine were obtained at the holding membrane potentials indicated to the left of each trace. Muscarine application is indicated by the arrowhead and vertical lines. (The duration of muscarine application was 2 s, with 500  $\mu$ M in the application pipette.) The order in which the measurements were made is given by the numbers at the right of the traces. Inward currents were observed positive to the reversal level, and outward currents at more negative potentials. The value of the reversal potential was determined by linear interpolation between the peak current changes found at each holding potential. B. The reversal potentials ( $V_{rev}$ ) were dependent upon  $[K^+]_{out}$ , shifting positive with elevation of  $[K^+]_{out}$ . The relationship between  $V_{rev}$  and  $[K^+]_{out}$  is plotted for 15 cells. The reversal potential shifted 58 mV positive per tenfold elevation of  $[K^+]_{out}$  (determined by least squares regression), implicating a  $K^+$  current in muscarinic responses. Modified from Ref. 32.

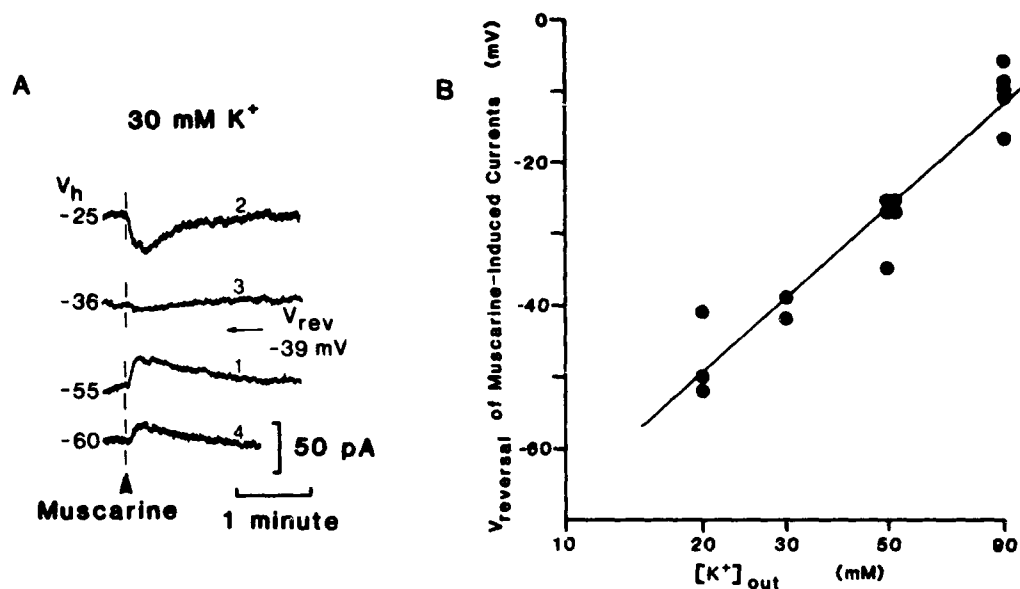


Fig. 3. Cholinergic response studied with the voltage-clamp technique illustrates the presence of M-current in a smooth muscle cell. The membrane potential was periodically hyperpolarized for two seconds to the levels indicated to the left of the voltage trace (upper trace). Membrane currents are shown in the lower traces, with inward currents downward. Currents caused by hyperpolarizing voltage jumps are shown on a faster time scale at the left. Upon hyperpolarization there was an initial rapid current jump ( $I_{initial}$ ) followed by a slow inward current relaxation to a steady-state level ( $I_{ss}$ ), which represents an outward K<sup>+</sup> current turning off. At the end of the two second pulse, there was a smaller rapid current jump, followed by an outward current relaxation to baseline, which is the K<sup>+</sup> current turning back on again (see diagram at left). The speed of the chart recorder was reduced during application of ACh (50  $\mu$ s in the application pipette), as indicated by the uppermost timing trace. ACh caused a net inward current at -25 mV, shown by the deflection of the current trace below the dashed line. The expanded trace at the center shows that in the presence of ACh, the current relaxations have been abolished and the initial rapid jump is smaller, the latter indicating a reduction in the conductance of the cell. The trace at right illustrates recovery after ~4 min. The experimental conditions were as described in Ref. 32, except that a discontinuous (switching) voltage-clamp amplifier (Axoclamp 2) was used.

single cells,<sup>32</sup> and we have shown that substance P can cause suppression of M-current in these cells.<sup>45</sup>

### Conclusions

The response of smooth muscle cells of the toad stomach to muscarinic agonists is remarkably similar to the response of sympathetic ganglia and other neurons – a K<sup>+</sup> conductance decrease. This demonstration of an M-current in smooth muscle provides an interesting addition to the categories of muscarinic effector mechanisms known to exist in different tissue types.

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## In-vivo labelling of peripheral muscarinic receptors

R. Hammer\*, H. Ladinsky and L. De Conti

*This study describes an atropine-sensitive binding component of intravenously administered  $^3\text{H}$ -N-methylscopolamine ( $^3\text{H}$ -NMS) in various peripheral tissues of the rat. Several lines of evidence indicate that this binding component reflects the specific interaction, in-vivo, of the radioligand with muscarinic receptors: i.e. 1. the specific binding exhibits a discrete tissue distribution, being observed only in organs different muscarinic organs is very low, being, in general, near  $0.5 \mu\text{g/kg i.v.}$ ; 2. the number of atropine saturable; 3. in agreement with the high affinity of NMS, the half saturation dose of  $^3\text{H}$ -NMS in different muscarinic organs is very low, being, in general, near  $0.5 \mu\text{g/kg i.v.}$ ; 4. the number of atropine sensitive binding sites is limited and does not exceed  $50 \text{ fmoles/mg wet weight}$  in any of the muscarinic organs investigated; 5. the specific binding is reversible with a time course similar to that seen in in-vitro binding studies; 6. it is inhibited dose-dependently by pretreatment with muscarinic antagonists; 7. in agreement with the pharmacological results, atropine exhibits a non-selective, and AF-DX 116, a new muscarinic receptor antagonist, a cardioselective inhibition profile. It is concluded that  $^3\text{H}$ -NMS is a very suitable in-vivo marker ligand for muscarinic receptors in peripheral organs. Potential applications of this technique are discussed.*

It is largely owing to *in-vitro* binding techniques that in the last decade rapid progress has been made in exploring the discrete distribution of muscarinic receptors and their densities in various organs, in discovering the existence of subtypes of muscarinic receptors and in characterizing allosteric muscarinic ligands. Nevertheless, there are certain limitations to this methodology which include a proteolysis reaction in certain protease-rich tissues during homogenization, the inadequacy of artificial buffers to simulate true physiological conditions and the loss of the extracellular/intracellular orientation of the membrane-bound receptors in broken cell preparations.

Many of the disadvantages of *in-vitro* binding can be avoided by *in-vivo* techniques. In this situation the specific interaction of ligand and receptor is determined after the ligand is injected into the living animal and the binding process takes place under true physiological conditions. In the past, rather extensive and detailed studies were reported of *in-vivo* binding in the CNS where the density of muscarinic receptors is much higher than in peripheral organs. In these studies, hydrophobic radioligands able to penetrate the blood-brain barrier were used.  $^3\text{H}$ -quinuclidinyl benzilate (QNB) was employed by Yamamura *et al.*<sup>1</sup>,  $^3\text{H}$ -dextimide by Laduron and Janssen<sup>2</sup> and  $^3\text{H}$ -scopolamine by Frey *et al.*<sup>3</sup> for the *in-vivo* labelling of muscarinic receptors in various brain regions. More recently, even external imaging of cerebral muscarinic receptors was reported with photon emission techniques using a radioiodinated QNB analog.<sup>4</sup>

With the exception of a few limited attempts,<sup>4,5</sup> no systematic investigations of the *in-vivo* labelling of muscarinic receptors in peripheral effector organs have been reported. This is surprising, since muscarinic receptors in smooth muscle organs in the heart and

exocrine glands are far better characterized pharmacologically than those in the CNS. A possible reason for this

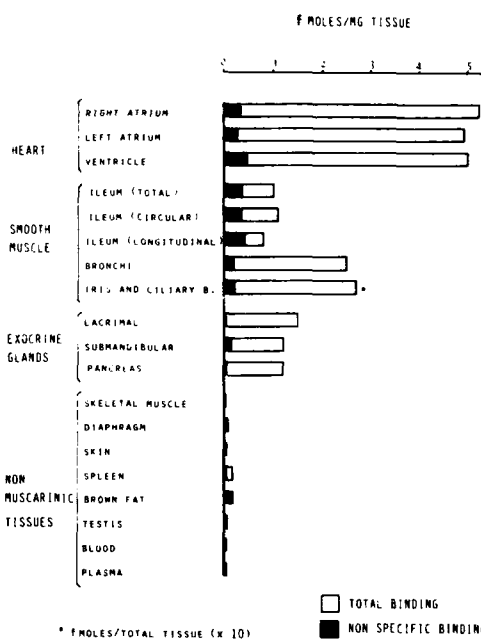


Fig. 1. Bound radioactivity in various muscarinic and non-muscarinic tissues of the rat measured 45 min after an i.v. dose of  $113 \text{ ng } ^3\text{H}$ -NMS/kg. Non-specific binding was determined in atropine pretreated animals ( $1 \text{ mg/kg i.v.}$  15 min prior to  $^3\text{H}$ -NMS). Each column represents the mean of 6 animals with the standard errors being less than 20% of the mean values.

\*FL Biochemistry, Boehringer-Ingelheim Zentrale GmbH, Ingelheim, FRG and Department of Biochemistry, Istituto De Angeli S.p.A., Milan, Italy.

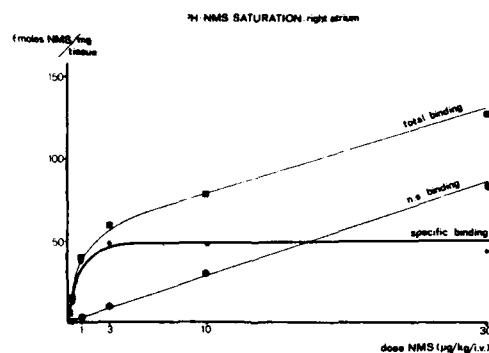


Fig. 2. Total, non-specific and specific binding of  $^3\text{H}$ -NMS in the right atrium as a function of increasing i.v. doses of the radioligand. Non-specific binding was assessed in atropine pretreated rats (1 mg/kg i.v. 15 min. prior to  $^3\text{H}$ -NMS). The data points represent the mean of 6 animals with a standard error of less than 10% of the mean values.

lies in the considerably lower densities of muscarinic receptors in peripheral organs compared to the brain. In addition, the radioligand most widely used *in vitro*,  $^3\text{H}$ -QNB, has one significant limitation: it does not label reliably exocrine glands *in vivo*, particularly the salivary and lacrimal glands (unpublished result from this laboratory). Therefore, in this study we have introduced  $^3\text{H}$ -N-methylscopolamine ( $^3\text{H}$ -NMS) as the radioligand of choice for *in-vivo* binding studies. This quaternary drug has only a weak tendency to partition into lipid membranes and to bind to non-specific sites and is a very suitable *in-vivo* marker ligand for muscarinic receptors in all relevant peripheral organs.

#### ***In-vivo* radioligand binding methodology**

Rats fasted overnight received, under light anesthesia, injections via the femoral vein of the radioligand  $^3\text{H}$ -NMS, saline and unlabelled antimuscarinic drugs depending on the type of experiment. For details concerning doses and time schedules of administration, see the appropriate section. Non-specific binding of  $^3\text{H}$ -NMS was assessed in animals pretreated with 1 mg atropine base/kg, i.v., a dose large enough to block virtually all the muscarinic receptors in peripheral organs.

For tissue sampling, the lightly anesthetized animals were rapidly bled through the inferior vena cava. The preparation of the organs (shown in Fig. 1) for radioactivity measurements, was performed as follows. The right and left atria, lacrimal glands, submandibular glands, skin, skeletal muscle (femoralis), diaphragm and brown fat were removed, weighed and transferred *in toto* to glass vials. The ventricle, pancreas, spleen and testes were minced into small pieces and homogenized in distilled water (25% w/w) with a Potter-Elvehjem teflon-on-glass homogenizer. Aliquots of the homogenates were pipetted into glass scintillation vials. To facilitate the isolation of the smooth muscles of the eye, the eye balls were frozen and carefully sectioned with a scalpel blade. The vitreous humour and the lens were discarded and the remaining tissue, containing both iris and ciliary body,

was transferred, without weighing, to a glass scintillation vial. To prepare the circular, longitudinal and total smooth muscle of the ileum, about 15 cm of the terminal small intestine were removed, rinsed through the lumen and then cut into two pieces of equal length. The distal part was used for assay of the total ileum (see below) and the proximal part was mounted on a thin glass pipette. The longitudinal muscle was stripped off with a wet cotton wisp, weighed and transferred to a glass scintillation vial. The intestinal tube was cut open and the mucosa was gently removed to yield the underlying circular muscle. The muscle was weighed and transferred to a glass scintillation vial. The distal part of ileum was opened and the intestinal mucosa was gently separated from remaining tissue (longitudinal and circular smooth muscle). The muscle tissue was weighed and transferred to a glass scintillation vial. For the preparation of the bronchi, the lungs were removed, the trachea discarded, and the parenchyma was very gently scraped away with a glass slide. The bronchial tree thus obtained was weighed and placed in a glass vial.

Whole organs or tissue homogenates were digested overnight under continuous shaking in a Dubnoff water bath at 50°C in the glass vials to which 1 ml Soluene had been added. To reduce quenching, the digested samples were oxidized with a mixture of 0.5 ml isopropanol and 0.2 ml 35% hydrogen peroxide. The samples were then mixed with 10 ml Dimilume, stored for 20 h to allow the decay of chemoluminescence and counted in a Tricarb scintillation spectrometer with an external standard control. Counts were corrected for quenching.

Non-linear least squares regression analysis of saturation data and time curves were performed with the TOPFIT pharmacokinetic package of Dr Karl Thomae, GmbH, Department of Biochemistry, Biberach, FRG.

#### ***In-vivo* labelling of muscarinic and non-muscarinic tissues with $^3\text{H}$ -NMS**

Fig. 1 shows the results of a study in which the *in-vivo* binding of  $^3\text{H}$ -NMS was investigated in muscarinic receptor- and non-muscarinic receptor-containing tissues. Briefly,  $^3\text{H}$ -NMS free base at a dose of 113 ng/kg was administered intravenously to rats pretreated either with saline or with 1 mg atropine base/kg i.v. The various organs were prepared for radioactivity measurement 45 min after the injection of  $^3\text{H}$ -NMS. The radioactivity content of tissues was defined as total binding and as non-specific binding in saline- and in atropine-pretreated animals, respectively.

It is evident that in all tissues known to contain muscarinic receptors, the pretreatment with atropine markedly inhibited the binding of  $^3\text{H}$ -NMS. This occurred in heart, smooth muscle organs and exocrine glands. In contrast, atropine pretreatment had no appreciable effect on  $^3\text{H}$ -NMS binding in non-muscarinic tissues: in skeletal muscle, diaphragm, skin, adipose tissue and testis the binding of  $^3\text{H}$ -NMS was virtually identical in animals with and without atropine pretreatment. These results suggest that the atropine suppressible part of the  $^3\text{H}$ -NMS binding is a measure of the specific interaction of the marker ligand with muscarinic receptors. The atropine insensitive component can be viewed as the equivalent of the non-specific tissue binding of  $^3\text{H}$ -NMS.

Several of the findings depicted in Fig. 1 deserve

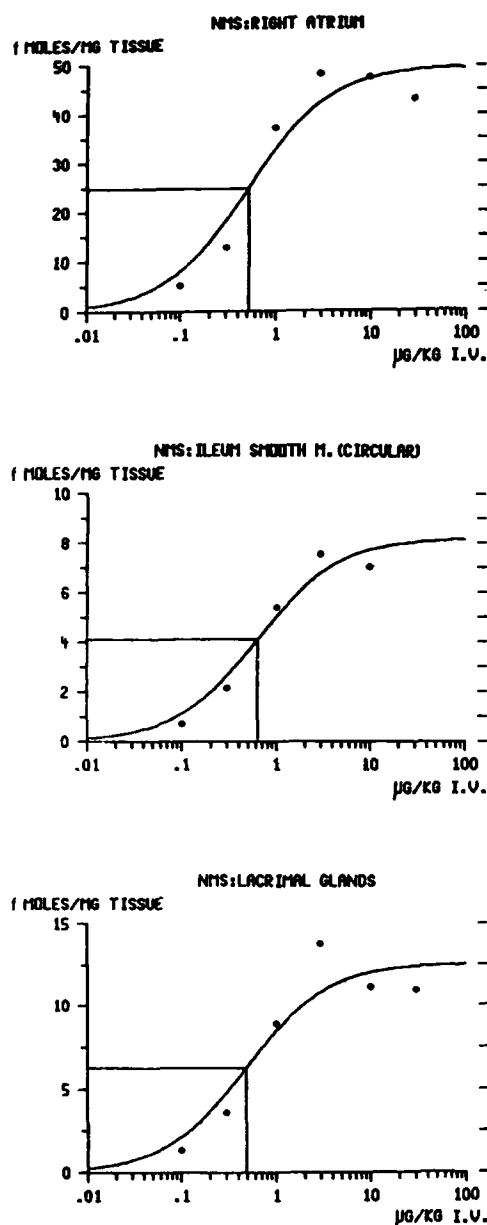


Fig. 3. Non-linear least squares analysis of the specific  $^3\text{H}$ -NMS saturation curves in the right atrium, ileum circular smooth muscle and lacrimal glands according to the one-site model. Upper panel: right atrium:  $D_{50} = 0.52 \mu\text{g/kg}$ ; receptor density = 49.8 fmoles/mg wet weight. Middle panel: ileum circular smooth muscle:  $D_{50} = 0.63 \mu\text{g/kg}$ ; receptor density = 8.2 fmoles/mg wet weight. Lower panel: lacrimal glands:  $D_{50} = 0.49 \mu\text{g/kg}$ ; receptor density = 12.6 fmoles/mg wet weight.

further comment. First, the relatively high *in-vivo* labeling of muscarinic receptors in the pancreas, iris and ciliary body, and bronchi is remarkable in view of the fact that, *in vitro*, it is technically difficult to carry out binding studies in these rat organs. Second, the low *in-vivo* binding in ileum smooth muscle was unanticipated. This result was unexpected particularly in the light of *in-vitro* binding studies in which the ileum longitudinal smooth muscle is known to contain the highest density of muscarinic receptors in the periphery. This apparent discrepancy between *in-vitro* and *in-vivo* binding in ileal longitudinal muscle is currently being investigated. Third, in the spleen, a 'non-muscarinic' organ, atropine inhibited a small, but significant proportion of the total  $^3\text{H}$ -NMS binding. This unexpected finding is, however, in line with a recent report describing the presence of presynaptic muscarinic receptors on sympathetic nerve fibers in this organ.<sup>6</sup>

#### *In-vivo* saturation curves with $^3\text{H}$ -NMS

In an attempt to determine the saturation behavior of  $^3\text{H}$ -NMS at muscarinic receptors in various effector organs,  $^3\text{H}$ -NMS, at doses ranging from 0.1 to 30  $\mu\text{g/kg}$ , was administered intravenously to rats pretreated either with saline or with 2 mg/kg atropine. The animals were killed 45 min after  $^3\text{H}$ -NMS injection, and the total binding (saline pretreatment) and the non-specific binding (atropine pretreatment) was determined in cardiac, smooth muscle and glandular tissues.

Fig. 2 depicts the full saturation data (total binding, non-specific binding and specific binding) for the rat right atrium. In this tissue, relatively high ratios of specific to non-specific binding were observed. It is notable that even at the highest dose of  $^3\text{H}$ -NMS (30  $\mu\text{g/kg}$ ), specific binding was still detectable. In general, conditions were less favorable in other tissues. Nonetheless, in all the tissues investigated (right atrium, ventricle, ileum circular smooth muscle, colon circular smooth muscle, bronchi, iris and ciliary body, lacrimal glands and pancreas), the specific saturation curves of  $^3\text{H}$ -NMS binding could be constructed. It was obvious from this series of experiments that the specific to non-specific binding ratio in a given tissue is primarily dependent on two parameters: the density of muscarinic receptors and the extent of non-specific binding.

The half saturation doses ( $D_{50}$  values) of  $^3\text{H}$ -NMS for muscarinic receptors in different organs were estimated empirically by non-linear regression analysis of the specific binding curves on the basis of a one-site model. Usually, calculated  $D_{50}$  values were around 0.5  $\mu\text{g/kg}$ . The greatest deviations were observed in the ventricle and in the iris and ciliary body in which the  $D_{50}$  values were 0.13 and 0.99  $\mu\text{g/kg}$ , respectively. Total receptor numbers were found to range from about 5.0 fmoles/mg wet wt (pancreas) to about 50 fmoles/mg wet wt (right atrium). Curves of best fit of the specific  $^3\text{H}$ -NMS binding in right atrium, ileum circular muscle and lacrimal gland are represented in Fig. 3.

#### *In-vivo* competition studies of atropine and AF-DX 116 v. $^3\text{H}$ -NMS

Atropine, in graded doses, given 15 min prior to a fixed dose of  $^3\text{H}$ -NMS (113 ng/kg, i.v.), inhibited the specific binding of the radioligand dose-dependently in all

**Table I.** Comparison of the i.v.  $ID_{50}$  doses of atropine and AF-DX 116 in inhibiting specific binding of  $^3H$ -NMS to several muscarinic effector organs.

Organ	$ID_{50}$ (mg kg i.v.)		Ratio
	atropine	AF-DX 116	
Right atrium	0.033	0.85	26
Ventricle	0.007	0.14	20
Ileum circular sm.m.	0.007	2.94	420
Iris and ciliary body	0.006	4.17	695
Lacrimal glands	0.021	~30.00	~1400
Submandibular glands	0.012	~30.00	~2500

The  $ID_{50}$  values represent the doses necessary to inhibit specific  $^3H$ -NMS binding by 50%, and were obtained from the semilogarithmic inhibition plots by linear regression. The data represent the means of 6 animals.

investigated muscarinic organs. This is indicative of a specific interaction with muscarinic receptors.  $ID_{50}$  values were estimated by linear regression from the semilogarithmic inhibition plots. As expected for a classical, non-selective drug, rather similar  $ID_{50}$  values, ranging from 6–33  $\mu$ g/kg i.v., were calculated for the different organs (Table I).

AF-DX 116, an analog of pirenzepine, which was designed and synthesized in the Chemistry Department at Thomae (Engel *et al.*, 7, 8), was also found to inhibit the specific binding of  $^3H$ -NMS in a dose-dependent manner. In contrast to atropine, AF-DX 116 exhibited selectivity, with the highest potency in the heart and the lowest in exocrine glands (Table I). When compared to atropine, AF-DX 116 was about 20–30 times weaker in cardiac tissues, whereas in smooth muscle organs and exocrine glands it was two to three orders of magnitude less potent than atropine. Thus, the cardio-selectivity of AF-DX 116, as originally demonstrated in isolated membranes<sup>9,10</sup> and pharmacological tests<sup>11,12</sup> is now confirmed by *in-vivo* binding studies. These results also support the concept of the existence of different muscarinic receptor subtypes in peripheral effector organs.<sup>9,10</sup>

#### Time course of *in-vivo* dissociation of $^3H$ -NMS from muscarinic tissues

We have been able to determine the *in-vivo* dissociation of  $^3H$ -NMS from various muscarinic tissues using the following protocol. Thirty min after the i.v. administration of  $^3H$ -NMS (113 ng/kg, i.v.) atropine was given by rapid intravenous bolus injection at a dose of 1 mg/kg. From a pharmacological viewpoint, this very high dose of atropine leads to a rapid occupation of all of the free muscarinic receptors in peripheral organs. As a consequence of this sudden depletion of free receptors, the formation of new  $^3H$ -NMS-receptor complexes is effectively blocked and the time course of the breakdown of the  $^3H$ -NMS-receptor complexes can be studied as a separate phenomenon.

Large differences in the dissociation behavior of  $^3H$ -NMS from different muscarinic organs were observed. In cardiac tissues (right atrium, left atrium, ventricle) the breakdown of the  $^3H$ -NMS-receptor complex was rapid; in smooth muscle organs (ileum circular muscle, iris and ciliary body) and in exocrine glands (lacrimal, submandibular, pancreas) it was slow. The terminal half-lives of the dissociation process estimated by non-linear curve fitting were approximately 2 min in cardiac tissues and

30–61 min in smooth muscle organs and in exocrine glands. As examples, Figs. 4 and 5 depict the fitted time

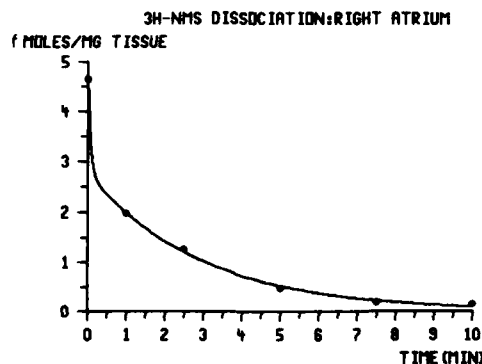


Fig. 4. Non-linear least squares analysis of the time course of *in-vivo*  $^3H$ -NMS dissociation in the right atrium according to a bi-exponential model. Each data point represents the mean of 6 animals. The terminal half-life was estimated to be 2.1 min.

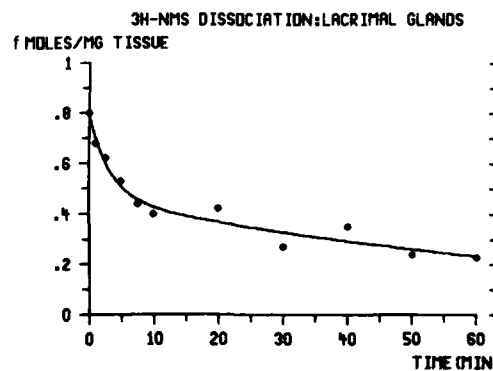


Fig. 5. Non-linear least squares analysis of the time course of *in-vivo*  $^3H$ -NMS dissociation in lacrimal glands according to a bi-exponential model. Each data point represents the mean of 6 animals. For the terminal half-life a value of 61.4 min was estimated.

curves of  $^3\text{H}$ -NMS dissociation from muscarinic receptors in right atrium and lacrimal glands.

The sharp contrast between the dissociation half-lives in cardiac tissues on the one hand and smooth muscles and glands on the other raises the question of the mechanism underlying this discrepancy. Two explanations may be considered. First, different subtypes of muscarinic receptors with a distinct tissue distribution may account for these results. If this explanation were to hold true, then it is surprising that the affinity estimates for  $^3\text{H}$ -NMS did not vary appreciably between the heart and the other organs both *in-vivo* (this paper) and *in-vitro*.<sup>13</sup> One must keep in mind, however, that the affinity of a ligand depends on both the dissociation and association rate constants and that a slow dissociation rate can be compensated by a small association rate constant (and vice versa). Second, the discrepancies in the  $^3\text{H}$ -NMS dissociation rates could also reflect tissue specific pharmacokinetics. A situation is conceivable in which the true dissociation rate of  $^3\text{H}$ -NMS from muscarinic receptors is similar in the different organs, but the kinetics of the loss of  $^3\text{H}$ -NMS depends largely on the tissue under investigation. It is possible to test the validity of the two hypotheses experimentally. If tissue specific pharmacokinetics is the underlying mechanism, then disrupting the tissues and studying the dissociation process in isolated membranes should eliminate the differences. On the other hand, if it is the true breakdown of the  $^3\text{H}$ -NMS-receptor complex which is measured *in vivo*, then differences in the dissociation should still be observed in broken cell preparations. It has been possible to determine experimentally which of the two explanations is the more valid.

Figure 6 illustrates the dissociation behavior of  $^3\text{H}$ -NMS from muscarinic receptors in broken cell preparations of two relevant organs: the heart and lacrimal gland. The striking dissimilarity of the  $^3\text{H}$ -NMS dissociation curves is immediately evident in these two *in-vitro* preparations. The data resemble the *in-vivo* situation in that the breakdown of the  $^3\text{H}$ -NMS-receptor complex in cardiac membranes is practically complete after 10 min (Fig. 4) and the dissociation of  $^3\text{H}$ -NMS has still not terminated after 45 min in the glandular preparation (Fig. 5).

These results indicate that it is the muscarinic receptor that determines the dissociation behavior of  $^3\text{H}$ -NMS and that different subtypes of muscarinic receptors exist in peripheral effector organs. The data also indicate that the classical antagonist NMS can discriminate between the different subtypes on the basis of its rate constants for the specific receptor interaction. These conclusions are in line with recent results using two different antagonists, hexahydrosiladifenidol<sup>14</sup> and AF-DX 116 (Refs 9, 10, 11, 12 and this paper) which are able to distinguish between

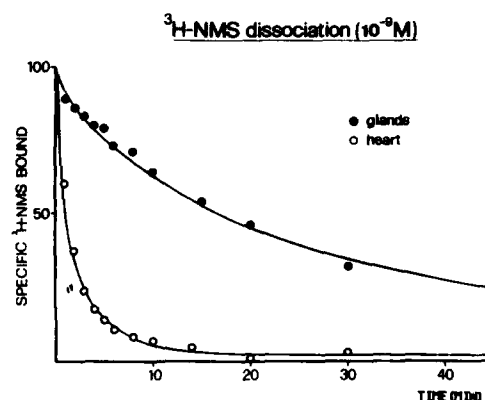


Fig. 6. In-vitro dissociation of  $^3\text{H}$ -NMS from crude membrane preparations of rat total heart and lacrimal glands. Experimental conditions as previously described.<sup>13</sup> The data are from one representative experiment in which each data point was measured in quadruplicate.

different end organ muscarinic receptors on the basis of affinity.

#### Conclusions and perspectives

From the experimental data reported here it is apparent that  $^3\text{H}$ -NMS is a suitable radioligand for studying the *in-vivo* binding to muscarinic receptors in peripheral effector organs. In the direct binding studies with this radioligand, *in vivo*, information has been obtained on the tissue distribution and densities of muscarinic receptors in different peripheral organs, while from the competition studies, the behavior of non-labelled ligands was determined indirectly and provided corroborative data on the potency and selectivity of muscarinic drugs.

In addition, given the fact that peripheral muscarinic effector organs, such as heart, smooth muscles and exocrine glands, are pharmacologically well characterized systems, there are many other uses for *in-vivo* binding studies (Table II). Particularly in the field of 'analytical pharmacology', there are several open and, in part, sophisticated questions to be resolved: can the concept of receptor reserve be substantiated *in vivo* by parallel investigations of *in-vivo* occupancies and pharmacological effects of full and partial muscarinic agonists? (It should be noted that  $^3\text{H}$ -NMS can be given at such low doses that it has no pharmacodynamic effect *per se*, yet the inhibition of specific  $^3\text{H}$ -NMS binding will reflect accurately the receptor occupancy of non-labelled, competing ligands). What relationship exists between the plasma

Table II. Potential topics for the application of *in-vivo* binding studies in muscarinic end organs

- The concept of receptor reserve (spare receptors)
- The relationship between plasma level, *in-vivo* receptor occupancy and pharmacological effect
- The presence of endogenous acetylcholine in muscarinic tissues
- The *in-vivo* regulation of receptor number
- The *in-vivo* behavior of allosteric drugs
- The *in-vivo* characteristics of agonist binding curves

level, the *in-vivo* receptor occupancy and the pharmacological effects for given muscarinic agonists and antagonists? Under normal physiological conditions, is there an appreciable fraction of the receptor population in certain tissues occupied by endogenously released acetylcholine? (We suspect, for example, that the consistently lower  $ID_{50}$  values in the ventricle as compared to the atria reflect the negligible parasympathetic innervation of the ventricle; see data in Table I.) Can the regulation of receptor number be studied under *in-vivo* conditions? Can gallamine slow the breakdown of the  $^3H$ -NMS receptor complex in the heart *in vivo*, as it does *in vitro*<sup>15</sup>? Does the *in-vivo* binding curve of carbachol in the heart extend over almost six log units *in vivo* as it does *in vitro*<sup>16</sup>?

It is anticipated that, in the future, carefully designed studies will contribute to our knowledge on many of the above mentioned problems.

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## Modulation of muscarinic receptors and their interactions

Mordechai Sokolovsky, Malca Cohen-Armon, Yaacov Egozi, David Gurwitz, Yoav I. Henis, Yoel Kloog, Etty Moscona-Amir and Gabriel Schreiber

*The binding properties of muscarinic receptors are modulated by interactions with other membrane proteins important for signal transduction. These include guanyl nucleotide binding proteins, voltage sensitive sodium channels, and calcium channels. The interaction of the muscarinic receptors with these membrane proteins, as well as with agonists and antagonists, are strongly affected by their conformational state. Individual agonists induce different conformational changes in the receptor. Cysteinylyl and tyrosyl residues may play an important role in the modulation of these conformational changes and interactions.*

Recent progress in the physiological and biochemical study of muscarinic receptors revealed that these receptors are affected by interactions with other receptors and/or with regulatory proteins (see Ref. 1, for discussion and relevant literature). We have previously proposed a general model for the interactions between the membrane components induced by agonists which are specific for different receptors. This model provides for modulation and regulation of signal transduction which distinguishes independent, simultaneous, and sequential actions of interacting agonists (Fig. 1). We make the assumption

We have speculated that X can be a guanyl nucleotide binding protein(s) (G-protein), ion channels, etc. Using this model we designed experiments to be summarized here that suggest: (i) In addition to G-protein(s), voltage-sensitive  $\text{Na}^+$ - and  $\text{Ca}^{2+}$ -channels modulate binding of agonist(s) to muscarinic receptors in certain tissues, and can act as component X in Fig. 1; (ii) Muscarinic agonists are capable of inducing different conformational changes in the receptor, a process which is dependent on the nature of the agonist under study; and (iii) Cysteinylyl and tyrosyl residue(s) play an important role in the modulation processes.

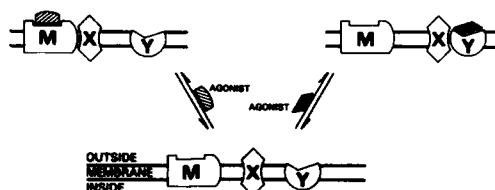


Fig. 1. Model depicting the putative interactions between the muscarinic system (M) and other receptor systems (Y).

that the muscarinic receptor (M) and another receptor/or membrane component (Y) are modulated via a common unit (X) that regulates both M and Y. At a given moment, X can be coupled either to M or to Y, but not simultaneously to both. Coupling of X to either M or Y will be recognized by the respective agonist as high-affinity state (M-X; Y-X), while the uncoupled situation (M; Y) will be characterized as low-affinity state with respect to agonist binding. Antagonists will bind similarly to the coupled and uncoupled state. A specific configuration of M...X...Y is typical for certain tissues but will vary between tissues. The fact that they have been detected in only a few cases so far,<sup>1</sup> might be basically related to technical problems of low signal to noise ratio.

### Muscarinic receptors and $\text{Na}^+$ - and $\text{Ca}^{2+}$ -channels

#### Interactions with agents affecting sodium channels

A recent study<sup>2</sup> raised the possibility that antiarrhythmic and local anesthetic drugs affect muscarinic receptors through a site analogous to the voltage-sensitive  $\text{Na}^+$ -channel. Preliminary experiments on the effects of batrachotoxin (BTX) on the muscarinic system lend further support to this suggestion (see Refs. 3 and 4 and references therein for reviews on  $\text{Na}^+$ -channels and neurotoxins). We therefore examined the effect induced by BTX on the binding of muscarinic ligands in four homogenates prepared from rat heart (atria and ventricle) and brain (brainstem and cortex). In all four preparations the binding of the tritiated antagonist ( $^3\text{H}$ )-N-methyl-4-piperidyl benzilate ([PH]-4NMPB) was similar in the presence or absence of  $1 \mu\text{M}$  BTX. On the other hand, the neurotoxin affected the displacement of [PH]-4NMPB by the muscarinic agonists carbamylcholine (CCh) and acetylcholine (ACh) in homogenates from atria, ventricle and brainstem, inducing a leftward shift in the displacement curves (Fig. 2). A similar effect (enhanced agonist affinity) was also observed in direct binding studies using [ $^3\text{H}$ ]ACh. No such effects were observed in cortex homogenates. Unlike the situation with CCh and ACh, no effects of BTX could be observed in any of the above homogenates with oxotremorine.

Analysis of the data according to a two-site model for agonist binding indicated that BTX did not change the relative proportions of high- and low-affinity agonist binding sites, but rather that it increased the affinity of the

Department of Biochemistry, The George S. Wise Faculty of Life Sciences, Tel-Aviv University, Tel-Aviv 69978, Israel.

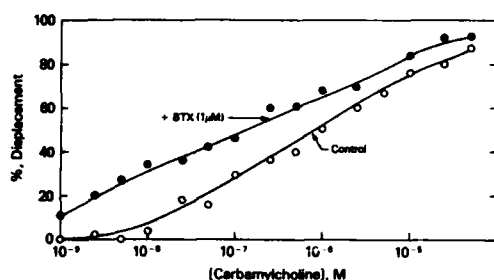


Fig. 2. Inhibition curves of the binding of 2 nM [ $^3$ H]-4NMPB by carbamylcholine in the atria preparation, in the absence and presence of 1  $\mu$ M batrachotoxin (BTX).

agonist toward the high-affinity site with insignificant effects on the binding to the low-affinity sites.<sup>5</sup> Thus, the  $K_H$  values calculated from the displacement of [ $^3$ H]-4NMPB by CCh in the absence and presence of 1  $\mu$ M BTX were  $(2.4 \pm 0.8) \times 10^{-7}$  M and  $(1.1 \pm 0.2) \times 10^{-7}$  M for the atria preparation, respectively, and  $(5.1 \pm 0.9) \times 10^{-7}$  M and  $(6.7 \pm 1.4) \times 10^{-8}$  M for the brainstem preparation. Certain other alkaloids, such as aconitine and veratridine, are known to interact with the same site on the  $Na^+$ -channel as BTX, although they are much less potent and efficacious (see Ref. 4 and references therein). Thus, if BTX affects the muscarinic receptors through binding to the  $Na^+$ -channel, its effects should be antagonized by these two drugs. Indeed, the effect of BTX on CCh binding to brainstem membrane preparations, was completely blocked by 10  $\mu$ M aconitine or 200  $\mu$ M veratridine. At these concentrations, the latter two drugs had no effect on either [ $^3$ H]-4NMPB or CCh binding in the absence of BTX. In contrast to aconitine and veratridine, 0.3  $\mu$ M tetrodotoxin (which acts at another site on the  $Na^+$ -channel) had no effect on either agonist or antagonist binding to muscarinic receptors in either the brainstem, atria or ventricle preparations.

The observation that BTX has no effect on antagonist binding indicates that direct competition by BTX for the muscarinic binding sites is highly unlikely. The latter conclusion is in agreement with the recent work of Daly and co-workers.<sup>4</sup> The effect of BTX on muscarinic agonist binding was observed at neurotoxin concentrations that are active in opening the voltage-sensitive  $Na^+$ -channels. Moreover, cinchocaine and tetracaine, two local anesthetic drugs, cancel the effect of BTX on the muscarinic receptors (the leftward shift of the agonist competition curve had disappeared) at a range of concentrations known to inhibit the activation of the  $Na^+$ -channel by BTX.

The observation that the interactions of BTX with the muscarinic receptors are detected in atria, ventricle and brainstem preparations, but not in the cortex, suggests either that they are region specific, or that they are undetectable in the cortex merely because the latter exhibits a much lower population of high affinity sites than the former two regions.

Another point that deserves attention is that the muscarinic agonists differ in their sensitivity to the BTX effect. Thus, while the binding of CCh and ACh is

modulated by the neurotoxin, oxotremorine binding in the same tissues is not affected. A possible explanation for this phenomenon is that oxotremorine binds to the muscarinic binding site in a different mode from CCh and ACh, a difference that is reflected in its lack of ability to sense the changes induced in the high-affinity state of the receptor by BTX. This interpretation is in accord with the discussion in the following sections as well as with recent kinetic studies in our laboratory,<sup>6</sup> which indicate that the interaction of oxotremorine (as reflected in the binding parameters) with the high-affinity sites is very different from that of CCh and ACh. In this context, one has to cite the recent report of Brown and Brown,<sup>7</sup> who observed major differences in the effects of CCh and oxotremorine on both cAMP formation and phosphoinositide hydrolysis in embryonic chicken heart cells. This promoted the suggestion that the receptor state associated with the inhibition of adenylate cyclase is the state common to the two agonists, while only CCh is associated with the phosphoinositide response.

#### Interactions with agents affecting calcium channels

We have previously demonstrated the presence of muscarinic receptors in rat adenohypophysis and described their biochemical characteristics.<sup>1</sup> Preliminary experiments indicated that  $Ca^{2+}$  can alter the characteristics of muscarinic binding in female rat adenohypophysis. We therefore investigated the effect of  $Ca^{2+}$  on the binding characteristics of [ $^3$ H]-4NMPB and oxotremorine in adenohypophyseal preparations of male and female rats at the various stages of the estrous cycle.<sup>8</sup> By using  $Ca^{2+}$  chelators such as EGTA, and  $Ca^{2+}$  channel blockers such as gallopamil (D-600), we showed that  $Ca^{2+}$  profoundly alters the binding characteristics of both antagonists and agonists to the muscarinic receptors. In female rats the effect of  $Ca^{2+}$  on antagonist binding is mainly on the maximal binding capacity of the receptors, while changes in the dissociation constants are much more moderate.

Table I. Effect of gallopamil (D-600) on binding of the muscarinic agonist oxotremorine in female rat adenohypophysis at the different stages of the estrous cycle, determined by competition with 2.0 nM [ $^3$ H]-4NMPB (means  $\pm$  SD).

Cycle stage	Test conditions	$\alpha$	$K_H(\mu M)$	$K_L(\mu M)$
Proestrus	+ $Ca^{2+}$ (1.9 mM)	80 $\pm$ 8	1.4 $\pm$ 0.5	6.0 $\pm$ 2.0
	- $Ca^{2+}$	35 $\pm$ 6	0.3 $\pm$ 0.2	3.5 $\pm$ 1.2
	+ $Ca^{2+}$ + D-600 <sup>b</sup>	40 $\pm$ 2*	0.2 $\pm$ 0.1	2.1 $\pm$ 1.0
Estrus	+ $Ca^{2+}$ (1.9 mM)	37 $\pm$ 7	0.2 $\pm$ 0.1	4.0 $\pm$ 1.0
	- $Ca^{2+}$	0	-	4.0 $\pm$ 0.2
	+ $Ca^{2+}$ + D-600 <sup>c</sup>	0	-	4.1 $\pm$ 0.1
Diestrus 2	+ $Ca^{2+}$ (1.9 mM)	30 $\pm$ 6	0.4 $\pm$ 0.4	2.4 $\pm$ 1.2
	- $Ca^{2+}$	77 $\pm$ 7	1.7 $\pm$ 0.8	8.5 $\pm$ 3.5
	+ $Ca^{2+}$ + D-600 <sup>d</sup>	85 $\pm$ 5*	1.8 $\pm$ 0.4	7.0 $\pm$ 1.5

$\alpha$  = Percentage of high-affinity binding sites for the agonist.

\*  $Ca^{2+}$  was omitted from the reaction mixture and 0.1 mM EGTA was added.

<sup>b</sup> Gallopamil was added to a final concentration of  $10^{-5}$  M.

<sup>c</sup> Gallopamil was added to a final concentration of  $4 \times 10^{-5}$  M.

<sup>d</sup> Gallopamil was added to a final concentration of  $10^{-4}$  M.

\*  $P < 0.005$  when compared to females at proestrus and diestrus 2 stages in the presence of  $Ca^{2+}$ . No significant difference was observed when compared to corresponding stages in the absence of  $Ca^{2+}$  (Student's *t* test).

Table II. Specific binding at equilibrium of [<sup>3</sup>H]-ACh and [<sup>3</sup>H]-4NMPB to muscarinic receptors in various rat brain regions and in cardiac atrium.

Tissue	No. of experiments	Specific binding of 25 nM [ <sup>3</sup> H]-4NMPB (fmol/mg protein)	Specific binding of [ <sup>3</sup> H]-ACh		[ <sup>3</sup> H]-ACh / [ <sup>3</sup> H]-4NMPB
			$B_{max}$ (fmol/mg protein)	$K_d$ (nM)	
Olfactory bulb	4	2860 ± 590	750 ± 185	32 ± 8	0.26 ± 0.04
Cerebral cortex	6	3020 ± 570	626 ± 138	34 ± 13	0.22 ± 0.03
Hippocampus	4	2845 ± 570	421 ± 88	73 ± 21	0.15 ± 0.02
Striatum	4	3244 ± 67	529 ± 127	55 ± 20	0.16 ± 0.04
Hypothalamus	3	1238 ± 290	402 ± 95	39 ± 14	0.33 ± 0.01
Brainstem	10	398 ± 112	176 ± 42	26 ± 8	0.48 ± 0.07
Cerebellum	4	196 ± 46	100 ± 29	19 ± 4	0.51 ± 0.06
Atrium	6	323 ± 90	209 ± 58	23 ± 7	0.71 ± 0.11

[<sup>3</sup>H]-ACh binding was determined using at least 11 concentrations in each curve (4–200 nM). The  $B_{max}$  and  $K_d$  values were calculated from Scatchard analysis. Specific binding of the antagonist [<sup>3</sup>H]-4NMPB was determined in incubations carried out at the same conditions, with 25 nM ligand, which saturates 98–99% of muscarinic receptors in all tissues studied.

The effect is expressed in the ability of  $Ca^{2+}$  to expose or to eliminate binding sites as a function of the estrous cycle. In agonist binding, the presence of  $Ca^{2+}$  has a pronounced effect on the proportion of high-affinity binding sites which parallels the changes induced in antagonist binding throughout the estrous cycle. As shown in Table I the main effect of  $Ca^{2+}$  appears to be on the exposure or elimination (depending on the stage of the cycle) of a population of binding sites which is mostly high-affinity with regard to oxotremorine binding. The  $Ca^{2+}$ -channel blocker, gallopamil, can completely block the effect of  $Ca^{2+}$  on the binding of both [<sup>3</sup>H]-4NMPB and oxotremorine (Table I). It should be noted that gallopamil by itself had no effect on the binding characteristics of [<sup>3</sup>H]-4NMPB, as shown by the fact that assays carried out in the adenohipophysis in the presence of gallopamil and absence of  $Ca^{2+}$  gave results similar to those obtained with the  $Ca^{2+}$ -free buffer. No differences were found in assays carried out with this blocker in preparations from the medulla-pons, where the  $Ca^{2+}$  effect is not exerted. The concentration of gallopamil required in order to induce such blocking also fluctuates during the estrous cycle. The cyclic variation in the effect of  $Ca^{2+}$  on gallopamil sites, as observed in competition experiments between gallopamil and [<sup>3</sup>H]-nitrendipine (Ref. 8 and references therein), is consistent with the cyclic blocking effect of gallopamil on  $Ca^{2+}$  channels with concomitant effect on the binding properties of adenohipophyseal muscarinic receptors as described above. It appears that the progression of the estrous cycle is accompanied by changes in the muscarinic receptors which may in turn be coupled to  $Ca^{2+}$ -channels. It will be of interest, of course, to investigate now the effect of  $Ca^{2+}$  on binding of other agonists (ACh, CCh) to this preparation.

#### High affinity binding of [<sup>3</sup>H]-ACh to muscarinic receptors

We have recently introduced a sensitive and specific procedure to assay the binding of [<sup>3</sup>H]-ACh to muscarinic receptors.<sup>9,10</sup>

The interaction of [<sup>3</sup>H]-ACh with the muscarinic receptor was studied in seven distinct rat brain regions and in heart atrium by employing 10  $\mu$ M atropine to define specific binding. The binding of [<sup>3</sup>H]-ACh to

various tissue homogenates shows the typical characteristics of specific interaction with high affinity between the ligand and muscarinic receptors. In all preparations examined (Table II), [<sup>3</sup>H]-ACh binding is saturable over the concentration range of 4–200 nM; the binding is reversible, and the labeled ligand can be displaced by muscarinic agonists and antagonists with the expected rank order of potency and stereospecificity; nicotinic or non-cholinergic drugs have no effect. Binding studies carried out under the experimental conditions employed in this work indicate that the interaction of the labeled ligand is with an apparently homogeneous population of high-affinity binding sites within each preparation. As shown in Table II, the ratio of muscarinic receptors labeled by [<sup>3</sup>H]-ACh to those labeled by the potent antagonist [<sup>3</sup>H]-NMPB varied markedly among tissues, from 0.15 in the hippocampus to 0.71 in the atrium. This ratio was lower in brain regions rich in muscarinic receptors, where a lesser sensitivity of [<sup>3</sup>H]-ACh binding to guanyl nucleotides was also observed.

Comparison of our data on [<sup>3</sup>H]-ACh binding to muscarinic receptors with the binding of other labeled muscarinic agonists reveals both similar and different features. The regional distribution of agonist binding sites is clearly similar. Thus, tissues or brain regions rich in [<sup>3</sup>H]-ACh binding sites also display a high density of oxotremorine-M ([<sup>3</sup>H]-Oxo-M) (Ref. 11) and of [<sup>3</sup>H](+)-cis-methylthiocholine ([<sup>3</sup>H]-CD) (Ref. 12) sites. However, the latter two drugs differ from the labeled neurotransmitter, [<sup>3</sup>H]-ACh, in two features: (a) In most tissues, curvilinear Scatchard plots are obtained for [<sup>3</sup>H]-Oxo-M and [<sup>3</sup>H]-CD binding in the nM range, while [<sup>3</sup>H]-ACh yields linear Scatchard plots at the wide concentration range employed (4–200 nM); (b) The  $B_{max}$  values derived from the direct binding experiments with labeled agonists are higher for [<sup>3</sup>H]-ACh than for the two synthetic agonists. The non-linear Scatchard plots of [<sup>3</sup>H]-Oxo-M and [<sup>3</sup>H]-CD could result from their binding to both super-high and high affinity sites, while [<sup>3</sup>H]-ACh binds to all high-affinity sites (both super-high and high) with a similar affinity. Thus, it is possible that muscarinic agonists may be capable of inducing different conformational changes in the receptor. Such a phenomenon was demonstrated earlier for the binding of muscarinic

antagonists (see Ref. 1 and references therein). The induction of different receptor conformations by different agonists is in line with the observations discussed above on differences in the modes of binding of oxotremorine and ACh and CCh as revealed by their binding kinetics; by the different sensitivities of their binding to modulation by batrachotoxin; by the earlier demonstration that oxotremorine differs from ACh and CCh in the effects on cAMP formation and phosphatidyl inositol hydrolysis; and by the responsiveness to chemical modifications (as discussed below).

#### Sensitivity to guanyl nucleotides

The sensitivity of [ $^3$ H]-ACh binding to guanyl nucleotides is most pronounced in those preparations enriched in agonist high-affinity binding sites, i.e. brainstem, cerebellum and atrium. The main effect of the guanyl nucleotides is reduction in the binding of [ $^3$ H]-ACh with minor changes in the corresponding  $K_d$  values.<sup>10</sup> This is entirely in agreement with previous findings derived from curves representing [ $^3$ H]-antagonist/agonist competition binding in the presence and absence of guanyl nucleotides (see Refs. 1 and 13 for reviews and references therein), and with a recent report employing [ $^3$ H]-Oxo-M (Ref. 14). In all brain tissues under investigation, the conversion of high-affinity to low-affinity sites induced by guanyl nucleotides is only partial, unlike in other receptors where a complete conversion has been observed.<sup>15</sup> Partial conversion of muscarinic receptors has also been reported by other laboratories, using [ $^3$ H]-Oxo-M (Ref. 14) or [ $^3$ H]-CD (Ref. 16). Thus, under the experimental conditions employed, the muscarinic sites detected by [ $^3$ H]-ACh binding can be divided into GTP-sensitive and GTP-insensitive sites. The former are most probably those sites that are reversibly coupled to a nucleotide binding protein(s). We have recently shown that the conversion of low- to high-affinity [ $^3$ H]-ACh sites which are GTP-insensitive may be induced by treatment for example of cerebral cortex or brainstem preparations with  $\text{Cu}^{2+}$  ions, probably via sulfhydryl groups on the receptor or on the guanyl nucleotide binding protein.<sup>17</sup> The reason for the induction of high affinity [ $^3$ H]-ACh sites which are insensitive to GTP is unknown, although preliminary experiments have indicated the possible role of SH/S-S transformation in such processes.<sup>17</sup> Finally, it should be noted that atrial and brain preparations reveal different sensitivities<sup>10</sup> to GTP or Gpp(NH)p ( $\text{I}_{50}$  of the Gpp(NH)p effect is  $0.3 \mu\text{M}$  v.  $1\text{--}6 \mu\text{M}$  in atria and in various brain regions, respectively). These different sensitivities could stem from different coupling of heart and brain muscarinic receptors with guanyl nucleotide binding proteins. Alternatively, they could indicate that the guanyl nucleotide protein designated  $G_i$  which transduces the inhibitory coupling of heart muscarinic receptors to adenylate cyclase, differs from the guanyl nucleotide binding protein which is coupled to brain muscarinic receptors, most likely the newly discovered family of the  $G_o$  (Ref. 18).

#### Chemical modification of functional groups in the muscarinic receptors

Chemical modification of functional groups in receptors for neurotransmitter and hormones can shed light on the structure of the receptor binding site(s). The binding properties of muscarinic receptors can be altered either by

blocking a sulfhydryl residue(s) or by sulfhydryl-disulfide transformations (see Ref. 19 for literature citation). For example, oxidation of SH residue(s) by the reagent diamide<sup>17</sup> induced interconversion of low-affinity to high-affinity sites in rat cortical preparation. However, the newly formed high-affinity sites are insensitive to guanyl nucleotide. A sulfhydryl reducing agent (DTT) was shown to be capable of inducing reduction of the disulfide formed as a consequence of the diamide treatment restoring the properties of the 'native' cortical muscarinic receptors. Low-affinity agonist sites can be converted into a GTP-sensitive high-affinity state by co-incubation with transition metal ions. However, as mentioned above, treatment with  $\text{Cu}^{2+}$  ion also induces conversion of low-affinity into high-affinity site(s) which are guanyl nucleotide insensitive. Thus, cysteinyl residue(s) are implicated to participate in the process of the transition of low- to high-affinity sites, the latter being guanyl nucleotide insensitive.

Tetranitromethane (TNM) has been shown to be a convenient reagent for the nitration of tyrosyl residues in proteins (see Ref. 19 and references therein). We therefore employed TNM in an effort to investigate whether tyrosyl residue(s) play a role in the binding of ligands to rat cortical muscarinic receptors. Modification with low TNM concentrations ( $100 \mu\text{M}$ , pH = 8.1, 20 min.) results in derivative(s) that essentially retain the native binding properties of antagonists but exhibit increased affinity (6.2 fold) towards several agonists such as ACh, CCh, arecoline and pilocarpine. For example [ $^3$ H]-ACh binding showed an 8-fold increase in affinity ( $K_d = 40 \pm 6 \text{ nM}$  for the control to  $K_d = 5 \pm 1.6 \text{ nM}$  for the modified receptors). No effect is seen when oxotremorine and/or Oxo-M are used as agonists. The effects of TNM treatment can be prevented by atropine or scopolamine ( $1 \mu\text{M}$ ), thus providing evidence that TNM modifies residue(s) at the binding sites. TNM is not totally selective for tyrosine. Previous studies have shown that sulfhydryl groups are potentially reactive toward TNM. A number of successive chemical modifications<sup>19</sup> were performed which indicated that the effect on agonists binding induced by TNM is not due to oxidation of a cysteinyl residue(s). Thus, a tyrosyl residue(s) is the most likely candidate as the residue responsible for the increased affinity of agonists to cortical muscarinic receptors. The modified receptors are insensitive to guanyl nucleotide treatment.

Also of interest is the observation that while antagonist(s) protect the muscarinic receptors from the chemical modification by TNM, agonists like ACh and/or CCh do not. One likely explanation is that these results stem from differences in the conformation of the receptor-ligand complex when agonists or antagonists are employed. Thus, in the antagonist-receptor complex the tyrosyl residue is not exposed to the reagent, while in the free receptor and in the agonist-receptor complex the tyrosyl residue is present in a position in which its microenvironment facilitates the chemical modification. In this context it is worth citing the recent report of Vanderheyden *et al.*<sup>20</sup> who suggested that agonists mediate a conformational change of the low-affinity state, resulting in their increased susceptibility towards *N*-ethylmaleimide alkylation. Since the muscarinic receptors may exist in both high- and low-affinity states for agonists, further experimentation with TNM in various

preparations – where the low-affinity state predominates (striatum and hippocampus) as well as in preparations rich in the high-affinity state (brainstem, heart atrium) will be needed in order to examine these suggestions.

In summary, binding of agonists to muscarinic receptors is modulated by interaction with membrane components such as guanyl nucleotide proteins, voltage-sensitive  $\text{Na}^+$ -channels, and  $\text{Ca}^{2+}$ -channels. Individual muscarinic agonists are capable of inducing different conformational changes in the receptor; these changes are determined by the nature of the agonist under study and might lead to different biochemical responses. Cysteiny and tyrosyl residue(s) may be differentially involved in such agonist-specific conformational changes.

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# The cholinergic hypothesis of dementia

Leslie L. Iversen

*Patients dying with senile dementia of Alzheimer's type have consistent damage to ascending cholinergic projections to cerebral cortex and hippocampus, and these changes are significantly correlated with the severity of the illness. The cortical cholinergic pathways are also damaged in patients with Parkinson's disease who exhibit clinical signs of dementia. Damage to the cortical cholinergic system in animals or treatment with cholinergic antagonists leads to impairments in learning and memory. Attempts to treat Alzheimer's dementia patients by administration of choline or lecithin have been largely unsuccessful, but some beneficial effects have been observed after treatment with cholinomimetics.*

## Cholinergic abnormalities in human brain in Alzheimer's disease and Parkinson's disease

There are now many reports of damage to cholinergic pathways in post-mortem brain samples from patients dying with Alzheimer's type dementia, and the subject has been extensively reviewed.<sup>1-3</sup> This brief article will describe only the salient features of the 'cholinergic hypothesis' of dementia. Although there are many changes in the brains of patients with Alzheimer's dementia, including loss of cells from cortex and sub-cortical areas, accumulation of neurofibrillary tangles and senile plaques, and damage to non-cholinergic systems,<sup>4</sup> the damage to ascending cholinergic projections to cerebral cortex and hippocampus appears likely to be of particular importance. These changes, reflected biochemically by a loss of the marker enzyme choline acetyltransferase (ChAT), are consistently found and are of considerable magnitude (50-70% reduction); furthermore, the extent of loss of ChAT activity from frontal and temporal cortices correlates significantly with the severity of the illness. This was shown originally by Perry *et al.*<sup>5</sup>, who correlated the cholinergic changes with pre-mortem dementia scores. In our own studies we observed significant correlations between the loss of cortical ChAT from frontal and temporal areas of cortex, and the numbers of senile plaques or silver staining neurofibrillary tangles counted in the contralateral areas of the same brains in 25 patients with Alzheimer's dementia.<sup>6</sup> The cortical changes in ChAT have been reported by some groups to parallel a loss of the subcortical cells of origin of the cortical cholinergic projection from the nucleus basalis of Meynert,<sup>7</sup> although others have observed little damage to this nucleus and argued that the cortical cholinergic changes may reflect a 'dying back' of the terminal processes of the cells.<sup>8</sup>

Several groups have observed that the cholinergic lesion is more severe in younger patients (dying before the age of 80 years) than in elderly subjects. In the Cambridge studies, involving some 50 patients with Alzheimer's dementia and a comparable number of normal elderly subjects, we found that the loss of ChAT from cortex was most profound in the younger patients (< 79 years), and

involved all cortical areas. The elderly sub-group of Alzheimer's patients (> 79 years) showed no significant loss of enzyme from any area of frontal cortex, although damage was still severe in temporal lobe and hippocampus.<sup>9</sup>

The loss of presynaptic cholinergic terminals from cerebral cortex and hippocampus in Alzheimer's dementia is not accompanied by any significant change in postsynaptic receptors, measured by radioligand binding in post-mortem tissue.<sup>10,11</sup> However, a more recent study suggests that binding sites of the M<sub>2</sub> muscarinic receptor sub-type may be significantly reduced in cortex in Alzheimer's dementia; these are greatly outnumbered by the predominant M<sub>1</sub> sub-type in cortex which are unaffected. The loss of M<sub>2</sub> sites may reflect the loss of receptors normally associated with the presynaptic cholinergic nerve terminals.<sup>12</sup>

The hypothesis that the damage to cortical cholinergic pathways may be responsible for the clinical syndrome of dementia in Alzheimer's disease is supported by the finding that similar changes occur in other neurodegenerative diseases in which dementia occurs. In particular it has become apparent that damage to ascending cholinergic projections is common in patients with Parkinson's disease, and that the severity of the cholinergic lesion reflects the presence or absence of clinical signs of dementia.<sup>13,14</sup> On the other hand, it has been argued that estimates of the incidence of dementia in Parkinsonian patients may be inflated,<sup>15</sup> suggesting the possibility that cholinergic damage may exist in some patients who do not exhibit any clear signs of cognitive impairment. As with the dopamine lesion in Parkinson's disease, however, the cholinergic deficit in dementia may well exhibit a 'threshold', so that partial damage will not necessarily be reflected by any functional impairment. Cholinergic abnormalities have also been reported in middle-aged patients with Down's syndrome, who exhibit severe intellectual impairment.<sup>16</sup>

## Animal models

The function of the ascending cholinergic projections to cortex and hippocampus is still only poorly understood. The neurones of origin of these pathways are diffusely distributed in basal forebrain and for this reason it has proved difficult to develop animal models in which these systems are selectively lesioned. Nevertheless, a number of groups have reported impairments of learning and

memory in rats in which the cortical cholinergic projection has been lesioned by injections of the neurotoxin ibotenic acid into the nucleus basalis.<sup>17,18</sup> It is well established that muscarinic antagonists, e.g. scopolamine, can disrupt short term memory and learning in animals,<sup>19</sup> and that cholinomimetics can act as alerting agents.<sup>20</sup> The ascending cholinergic projections appear to be involved in alerting or arousal functions that are crucial for the establishment of short term memory.

Aged laboratory animals, on the whole, do not seem to offer useful models for studies of the 'cholinergic hypothesis'. The elderly rat exhibits impaired ability to learn and remember simple avoidance tasks, but these deficits are not reversed by treatment with cholinomimetic drugs. The aged primate, however, represents a more interesting model. Bartus and his colleagues<sup>21</sup> have shown that elderly Rhesus monkeys exhibit a profound deficit in short term memory which can be reversed partly by treatment with carefully titrated doses of physostigmine.

#### Pharmacological manipulation of cholinergic systems

Human subjects show marked impairments of short term memory and cognitive function after treatment with the muscarinic antagonist drug scopolamine,<sup>22</sup> and the symptoms of this 'scopolamine dementia' are largely reversed by physostigmine.<sup>22</sup>

The cholinergic hypothesis of dementia has suggested that treatment with cholinergic agents might be of therapeutic benefit, and this has stimulated a number of clinical trials. Many attempts have been made to replace acetylcholine by treatment with precursor choline, usually administered in the form of lecithin (for review see Refs 23, 24), but the results have generally been negative, although a recent study with an elderly sub-group of subjects reported some positive benefit after such treatment.<sup>25</sup> More encouraging results have been obtained with physostigmine or cholinergic agonists, although the therapeutic benefits seen were generally modest and transient, and it was not always possible to identify a therapeutic window to avoid the numerous unpleasant side-effects of such drugs.<sup>24</sup> Improvements in cognitive and social function have also been reported in four Alzheimer's patients who received chronic intracranial infusions of the cholinergic agonist bethanechol by means of an implanted infusion system.<sup>26</sup> These preliminary findings offer some encouragement to the hope that the cholinergic hypothesis of dementia may eventually lead to the development of rational and effective therapies.

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## Biochemical and functional basis of putative muscarinic receptor subtypes and its implications

Mark Watson, William R. Roeske, Thomas W. Vickroy,  
Thomas L. Smith, Kazufumi Akiyama, Karoly Gulya,  
Sue P. Duckles, Mariangela Serra, Abdu Adem, Agneta Nordberg,  
Donald R. Gehlert, James K. Wamsley and Henry I. Yamamura

*The advent of pirenzepine (PZ) has generated considerable interest in the basis and the implications of muscarinic acetylcholine receptor (mAChR) heterogeneity. [<sup>3</sup>H]PZ has been used extensively to identify and characterize the putative M<sub>1</sub> mAChR subtype, for which this ligand has high affinity. Autoradiographic studies have been carried out to localize binding sites precisely for the muscarinic agonist [<sup>3</sup>H](+)-cis-methyldioxolane ([<sup>3</sup>H](+)-CD), for the sodium-dependent high affinity choline uptake blocker hemicholinium-3 ([<sup>3</sup>H]HC-3), for the M<sub>1</sub> selective antagonist [<sup>3</sup>H]PZ and for [<sup>3</sup>H](−)-quinuclidinyl benzilate ([<sup>3</sup>H](−)-QNB), which labels M<sub>1</sub> and M<sub>2</sub> mAChR subtypes in various tissues with relatively equal affinity. Interestingly, both putative mAChR subtypes appear to undergo axonal transport. Therapeutic and adverse effects of muscarinic drugs may be mediated by independent mAChR subpopulations which may be pharmacologically exploited to produce highly selective and efficacious new drugs.*

Despite the existence of some evidence to the contrary, muscarinic cholinergic responses to acetylcholine (ACh) were widely believed to be mediated by a single population of membrane receptors differing only in their regulatory properties and effector coupling mechanisms until it was recently shown that pirenzepine (PZ) selectively interacts with functional muscarinic receptors as well as mAChR binding sites in membrane preparations. Physiological studies with PZ show significant differences from the classical antimuscarinic drug atropine.<sup>1,3</sup> Pirenzepine was shown to antagonize excitatory ganglionic mAChR with a nearly 40-fold greater potency than its antagonism of atrial mAChR, while atropine was equipotent. This tissue-specific action of PZ is well supported by indirect binding studies which show differences in the numbers of high and low affinity PZ binding sites in the brain and other tissues.<sup>4</sup> These initial results have been confirmed and extended in homogenates by direct studies of [<sup>3</sup>H]pirenzepine ([<sup>3</sup>H]PZ) binding.<sup>5,15</sup>

### Heterogeneity evidenced by pirenzepine

The use of [<sup>3</sup>H]PZ as a radioligand for muscarinic receptor binding sites was first reported in 1982<sup>6</sup> and has since been used to study the unique binding properties of muscarinic receptors in different tissues.<sup>6,7</sup> Radioligand binding studies show that [<sup>3</sup>H]PZ labels a high affinity subpopulation of [<sup>3</sup>H]quinuclidinyl benzilate ([<sup>3</sup>H](−)-QNB) sites which demonstrate considerable tissue differences but maintain a typical muscarinic pharmacological profile.<sup>6</sup> Further, results from [<sup>3</sup>H]PZ membrane binding studies correlate well with studies in whole animals which

demonstrate: (i) a large proportion of high affinity [<sup>3</sup>H]PZ sites (relative to [<sup>3</sup>H](−)-QNB) in tissues<sup>5,7</sup> sensitive to the antimuscarinic action of PZ,<sup>3</sup> and (ii) a very low proportion of high affinity [<sup>3</sup>H]PZ sites in tissues where PZ seems to have little effect upon cholinergic transmission. High densities of high affinity [<sup>3</sup>H]PZ binding sites (M<sub>1</sub> sites) have been detected in homogenates of cerebral cortex,<sup>5,6</sup> corpus striatum and hippocampus while low-affinity [<sup>3</sup>H]PZ binding sites (M<sub>2</sub> sites) predominate in homogenates of cerebellum and other areas of the central nervous system (CNS).<sup>5,6,10,15</sup> and peripheral tissues such as the ileum and heart.<sup>5</sup> Recent data from developmental studies in the murine cerebral cortex and heart<sup>14</sup> show these differences exist at birth and persist throughout postnatal ontogeny. The K<sub>i</sub> values for various agonists and antagonists obtained in studies of cerebral cortical and cardiac homogenates labeled with [<sup>3</sup>H]PZ, [<sup>3</sup>H](−)-QNB, or [<sup>3</sup>H](+)-cis-methyldioxolane ([<sup>3</sup>H](+)-CD) are all very similar and in good agreement with the range of affinity values obtained for each of these compounds (with the exception of agonists versus the highest affinity state labeled by [<sup>3</sup>H](+)-CD) in a variety of functional studies in various tissues.<sup>15</sup> Pirenzepine emerges as a unique compound possessing a wide range of affinity values in both binding and functional studies. The relative selectivity of antagonists appears to be unrelated to the guanine nucleotide binding protein.<sup>15</sup> Thus, as we have speculated previously,<sup>5,11,15</sup> PZ's selectivity does not appear to be dependent upon coupling to either a guanine nucleotide binding protein or to the low affinity agonist state. In fact we have recently demonstrated that potent classical muscarinic agonists, such as carbachol (CCh), display a quantitatively small (compared to the heart), but reproducible shift to lower affinity on M<sub>1</sub> cerebral cortical mAChR labeled with [<sup>3</sup>H]PZ (Watson *et al.*, submitted).

Departments of Pharmacology and Internal Medicine, College of Medicine, University of Arizona Health Sciences Center, Tucson, AZ 85724, USA.

Table 1. Autoradiographic localization of cholinergic binding sites in the CNS

Location	[ <sup>3</sup> H]HC-3 <sup>a</sup>	[ <sup>3</sup> H](+)-CD <sup>b</sup>	[ <sup>3</sup> H](−)-QNB <sup>c</sup>	[ <sup>3</sup> H]PZ <sup>d</sup>
Accessory optic nuclei	3.68 ± 0.5	—	—	—
Amygdala (lateral nucleus)	4.07 ± 0.2	0.098 ± 0.02	—	—
Basal forebrain	1.69 ± 0.1	—	—	—
Caudate-putamen	6.96 ± 0.2	0.84 ± 0.05	64.0 ± 2.3	64.3 ± 0.9
Cerebellum (molecular layer)	—	—	7.4 ± 1.1	9.2 ± 0.6
(granular cell layer)	—	0.146 ± 0.003	19.4 ± 2.1	9.4 ± 1.5
Corpus callosum	0.02 ± 0.03	—	—	—
Cortex (parietal) lam I–III	—	0.062 ± 0.01	44.4 ± 2.0	42.9 ± 0.5
lam IV	—	0.330 ± 0.04	43.5 ± 1.0	35.6 ± 1.9
lam V	—	0.021 ± 0.005	34.6 ± 0.7	33.6 ± 0.7
lam VI	—	0.280 ± 0.03	39.9 ± 0.8	40.0 ± 0.7
Cortex (temporal) lam I–III	0.15 ± 0.07	—	—	—
lam IV	0.53 ± 0.3	—	—	—
lam V–VI	0.13 ± 0.09	—	—	—
Dentate gyrus (hilus)	1.68 ± 0.1	—	—	—
(molecular layer)	1.05 ± 0.2	—	60.2 ± 0.3	60.6 ± 0.7
(granule cell layer)	4.69 ± 0.2	—	—	—
Facial nucleus	—	2.80 ± 0.07	31.8 ± 1.6	1.3 ± 2.0
Globus pallidus	0.03 ± 0.04	0.098 ± 0.02	7.0 ± 1.3	9.2 ± 1.1
Habenula (lateral nucleus)	4.12 ± 0.3	—	—	—
(medial nucleus)	4.30 ± 0.1	—	—	—
Hippocampus, stratum oriens	1.20 ± 0.1	0.140 ± 0.02	60.3 ± 0.6	59.7 ± 0.8
stratum radiatum	0.50 ± 0.06	0.006 ± 0.002	59.9 ± 0.5	60.6 ± 0.4
stratum lacunosum-moleculare	0.72 ± 0.06	—	55.3 ± 0.5	55.7 ± 0.5
Hypoglossal nucleus	—	3.99 ± 0.27	39.4 ± 0.3	15.1 ± 1.3
Hypothalamus (dorsal lat. n.)	1.41 ± 0.1	—	—	—
Islets of Cajal	—	1.19 ± 0.2	—	—
Lateral septum	1.78 ± 0.2	—	—	—
Lateral mammillary nucleus	1.19 ± 0.1	—	—	—
Medial geniculate nucleus	0.63 ± 0.2	—	—	—
Nucleus accumbens	4.91 ± 0.1	0.34 ± 0.05	48.0 ± 1.8	49.4 ± 0.5
Nucleus tractus solitarius	—	4.68 ± 0.3	28.0 ± 1.6	4.7 ± 2.2
Olfactory tubercle	5.24 ± 0.3	0.032 ± 0.002	—	—
Periaqueductal gray matter	1.99 ± 0.2	0.21 ± 0.02	—	—
Spinal cord (cerv) Dorsal horn	—	0.099 ± 0.004	22.7 ± 1.0	23.4 ± 0.9
Ventral horn	—	0.019 ± 0.001	25.8 ± 1.6	4.0 ± 1.2
Substantia nigra, zona compacta	0.84 ± 0.1	—	—	—
zona reticulata	0.63 ± 0.2	—	—	—
Superior colliculus	1.52 ± 0.2	—	43.3 ± 0.3	13.2 ± 1.2
deep	—	0.73 ± 0.02	—	—
superficial	—	1.03 ± 0.1	—	—
Supramammillary nucleus	3.59 ± 0.2	—	—	—
Thalamus, central nucleus	1.58 ± 0.1	—	—	—
dorsal lateral nucleus	0.58 ± 0.06	—	—	—
dorsal medial nucleus	2.10 ± 0.1	—	—	—
ventral posterior lat. n.	0.87 ± 0.2	—	—	—
ventral posterior med. n.	0.26 ± 0.1	—	—	—
lateral nucleus	—	0.24 ± 0.02	—	—
paraventricular nucleus	—	0.52 ± 0.05	—	—
ventral posterior nucleus	—	0.26 ± 0.02	—	—
Tractus diagonalis	—	—	32.0 ± 3.0	13.8 ± 2.3

<sup>a</sup> Values of [<sup>3</sup>H]HC-3 binding in the table represent the mean ± S.E. in fmol/mg tissue. Assays were conducted in phosphate buffered salt solution containing 2.5 nM [<sup>3</sup>H]HC-3 for 30 min at 25 °C, as previously described.<sup>31</sup>

<sup>b</sup> Values of [<sup>3</sup>H](+)-CD binding represent the mean ± S.E. in fmol/mg tissue. Assays were conducted in 10 mM Na-K phosphate buffer containing 5 nM [<sup>3</sup>H](+)-CD for 2 h at 0 °C, as previously described.<sup>30</sup>

<sup>c</sup> Values of [<sup>3</sup>H](−)-QNB binding represent the mean ± S.E. autoradiographic grain density by a Stahl Industries Computer (n=4–6). Assays were done in Krebs phosphate buffer with 1 nM [<sup>3</sup>H](−)-QNB for 2 h at 25 °C, as previously described.<sup>32</sup>

<sup>d</sup> Values of [<sup>3</sup>H]PZ binding represent the mean ± S.E. autoradiographic grain density by a Stahl Industries Computer (n=4–6). Assays were done in Krebs phosphate buffer containing 20 nM [<sup>3</sup>H]PZ for 1 h at 25 °C, as previously described.<sup>32</sup>

\* Values are not directly comparable since ligands were of different specific activities and were incubated for different time periods.

While PZs discriminatory capabilities have been widely interpreted within the  $M_1$ ,  $M_2$  framework originally proposed by Goyal and Rattan,<sup>16</sup> the presence of high affinity ( $M_1$ ) and low affinity ( $M_2$ ) [ $^3$ H]PZ binding sites may be somewhat oversimplified. For example, it is possible that high affinity and low affinity [ $^3$ H]PZ binding sites are not homogeneous and that more than two subtypes exist.<sup>17,18</sup> On the other hand there is evidence to suggest that there exists only one receptor protein. A recent report by Roeske and Venter,<sup>19</sup> for example, suggested that [ $^3$ H]PZ and [ $^3$ H](+)-QNB may bind to separate regions of the receptor domain in solubilized rat brain preparations. Yet similar studies of [ $^3$ H]PZ and [ $^3$ H](+)-QNB binding to membrane preparations in rat cerebral cortex and heart revealed little difference in time courses of decay of the drug-receptor complex of these ligands at 0°C, 25°C, or 37°C (Watson *et al.*, submitted). The methods, results and conclusions of additional solubilization studies are also different.<sup>20,21</sup> While the pharmacological relationship between solubilized and membrane-bound receptor sites is not clearly defined, this difference may help to explain the unique regulatory profile of high-affinity [ $^3$ H]PZ binding relative to [ $^3$ H](+)-QNB.<sup>6,11,13</sup> Indeed, the unique regulatory profile of PZ should be carefully considered when attempting to carry out comparative studies of high affinity [ $^3$ H]PZ and [ $^3$ H](+)-QNB binding (Watson *et al.*, submitted). The ionic composition of the buffer, specifically concentrations of  $Na^+$ ,  $K^+$ , or  $Mg^{2+}$ , alters [ $^3$ H]PZ binding while having minimal effects on [ $^3$ H](+)-QNB binding.<sup>6,13</sup> Therefore, in a low ionic strength buffer, high affinity [ $^3$ H]PZ binding sites are more numerous (relative to [ $^3$ H](+)-QNB sites) whereas the ratio of [ $^3$ H]PZ sites/[ $^3$ H](+)-QNB sites would be lower in a more physiological buffer. Thus, the percentage of  $M_1$  sites can be influenced by ionic composition of buffers. While the precise nature of [ $^3$ H]PZ binding is not yet understood, it is the most selective radiolabeled muscarinic antagonist now available.

#### Heterogeneity detected by agonists

Using a newly developed rapid filtration binding assay,<sup>22,23</sup> we have also studied the binding of unlabeled PZ in tissues labeled with [ $^3$ H](+)-CD, a compound known as a potent muscarinic agonist in studies of the guinea-pig ileum. Inhibition curves for PZ versus [ $^3$ H](+)-CD labeled membranes are flat (Hill values < 1) in both heart and cerebral cortex. Moreover, PZ has four times higher affinity for the extremely high affinity ( $K_d = 2$  nM) agonist sites in the cerebral cortex as compared to the heart. Analysis of these data by a two-site binding model shows a significantly improved fit of the curves. In the cerebral cortex, 80% of the sites labeled by [ $^3$ H](+)-CD have high affinity for PZ ( $IC_{50} = 60$  nM) with the rest showing a 20-fold lower affinity. Yet, in the heart, 28% of [ $^3$ H](+)-CD labeled sites show high affinity for PZ ( $IC_{50} = 21$  nM) with the majority of sites showing low affinity for PZ. In contrast, other classical muscarinic antagonists show little difference in comparisons of cerebral cortical and cardiac mACHR sites labeled with [ $^3$ H](+)-CD. Pirenzepine's ability to selectively interact with mACHR subtypes when labeled with a [ $^3$ H]agonist ligand is remarkable since it not only verifies the data obtained by labeling with a [ $^3$ H]antagonist, but also

suggests further complications regarding the relationship between mACHR heterogeneity sensed by agonists and that sensed by antagonists.<sup>6,11</sup>

We have previously noted that the mACHR heterogeneity sensed by agonists and antagonists does not appear to be the same.<sup>6,10,11,15</sup> Inhibition studies of muscarinic agonists versus [ $^3$ H]PZ labeled mACHR binding sites in the rat cerebral cortex yielded Hill values less than one, which implies the existence of multiple agonist affinity states within the binding sites which show high affinity for [ $^3$ H]PZ (Ref. 6). Shallow agonist [ $^3$ H]-antagonist competition curves have been well described previously and agonist heterogeneity was believed to reflect three separate states of one muscarinic receptor.<sup>24,26</sup> However, recent studies reveal additional affinity states and indicate that the affinities and relative proportions of states are highly dependent upon the composition of the buffered media and the presence of various regulators.<sup>11,23,27,29</sup> Moreover, the highest affinity agonist state in the rat cerebral cortex and heart also show different structure-binding relationships among mACHR in these tissues.<sup>11,22,23,29</sup> Thus, PZ and other drugs revealed differences in mACHRs even when only the highest affinity agonist state is labeled. Also, while low affinity agonist states predominate in the cerebral cortex where [ $^3$ H]PZ binds most sites with high affinity, higher affinity agonist states dominate in the cerebellum where there is minimal [ $^3$ H]PZ binding. Yet most ileal sites have both low affinity for agonists and minimal [ $^3$ H]PZ binding. Moreover, when the cerebral cortical high affinity [ $^3$ H]PZ site ( $M_1$ ) is selectively labeled, agonists produce Hill values of less than one, reflecting the presence of both a higher and relatively lower affinity agonist state.<sup>6</sup> Since most ligands appear to show some potential selectivity for agonist states and  $M_1$  and  $M_2$  subtypes,<sup>6,11</sup> concern for the relative selectivity for each type of heterogeneity is necessary to assure proper interpretation of these complexities.<sup>11</sup>

#### Autoradiographic localization of putative mACHR subtypes

We have been conducting extensive light microscopic autoradiographic studies<sup>12,30,34</sup> to visualize agonist as well as  $M_1$  and  $M_2$  mACHR subtypes within various tissues, since the precise localization of subtypes may be crucial to the eventual elucidation of specific functions selectively mediated by each of these mACHR subtypes but cannot be determined from gross anatomical studies in homogenates. In the CNS (Table I), comparisons of autoradiograms of [ $^3$ H]QNB binding (labeling  $M_1$  and  $M_2$  sites) with [ $^3$ H]PZ binding (labeling  $M_1$  sites) show distinctly different labeling patterns.

In order to further characterize the relationship of putative  $M_1$  and  $M_2$  mACHR subtypes to the highest affinity state for agonists and the relationship to pre- and post-synaptic receptors, we have conducted comparative autoradiographic studies of the binding of [ $^3$ H]-hemicholinium-3 ([ $^3$ H]HC-3), [ $^3$ H](+)-CD, [ $^3$ H]PZ and [ $^3$ H](+)-QNB to slices of the rat CNS. These data from thaw-mounted frozen tissue slices are summarized in Table I. Each of these ligands possesses its own discrete localization. It is evident that [ $^3$ H]PZ labels a discrete subpopulation of sites when compared to [ $^3$ H](+)-QNB binding site densities in these various CNS regions. [ $^3$ H]PZ labels regions of the cerebral cortex, hippo-

campus, striatum and the dorsal horn of the spinal cord equally with [ $^3$ H](−)QNB. The cerebellum, nucleus tractus solitarius, facial nucleus, and ventral horn of the spinal cord are all far better labeled with [ $^3$ H](−)QNB.

Interestingly, as was also seen in homogenates,<sup>11</sup> high affinity [ $^3$ H](+)CD binding reveals a discrete regional distribution of its own. Many have speculated regarding the possible relationship between the reciprocal correlation of the high affinity agonist state and PZ binding. The correlation of apparent binding site densities for [ $^3$ H](+)CD and [ $^3$ H](−)QNB is very poor ( $r = -0.3$ ). However, these results are in agreement with our finding of a wide range of values for the percentage of high

affinity agonist sites for [ $^3$ H](+)CD in various CNS regions.<sup>11</sup> In contrast, there is a good inverse correlation ( $r = -0.8$ ) between the regional density of [ $^3$ H](+)CD and [ $^3$ H]PZ binding sites (Table 1). Yet, many areas provide notable exceptions to this rule. For example, the ventral horn of the spinal cord contains few high affinity [ $^3$ H](+)CD sites or [ $^3$ H]PZ binding sites and the caudate-putamen contains large numbers of both [ $^3$ H]PZ and [ $^3$ H](+)CD sites. Thus, while an inverse relationship exists in certain discrete CNS areas, it would appear to be an oversimplification to suggest agonist affinity states are the basis for the apparent heterogeneity sensed by PZ.

Regions of intense [ $^3$ H]HC-3 labeling include the

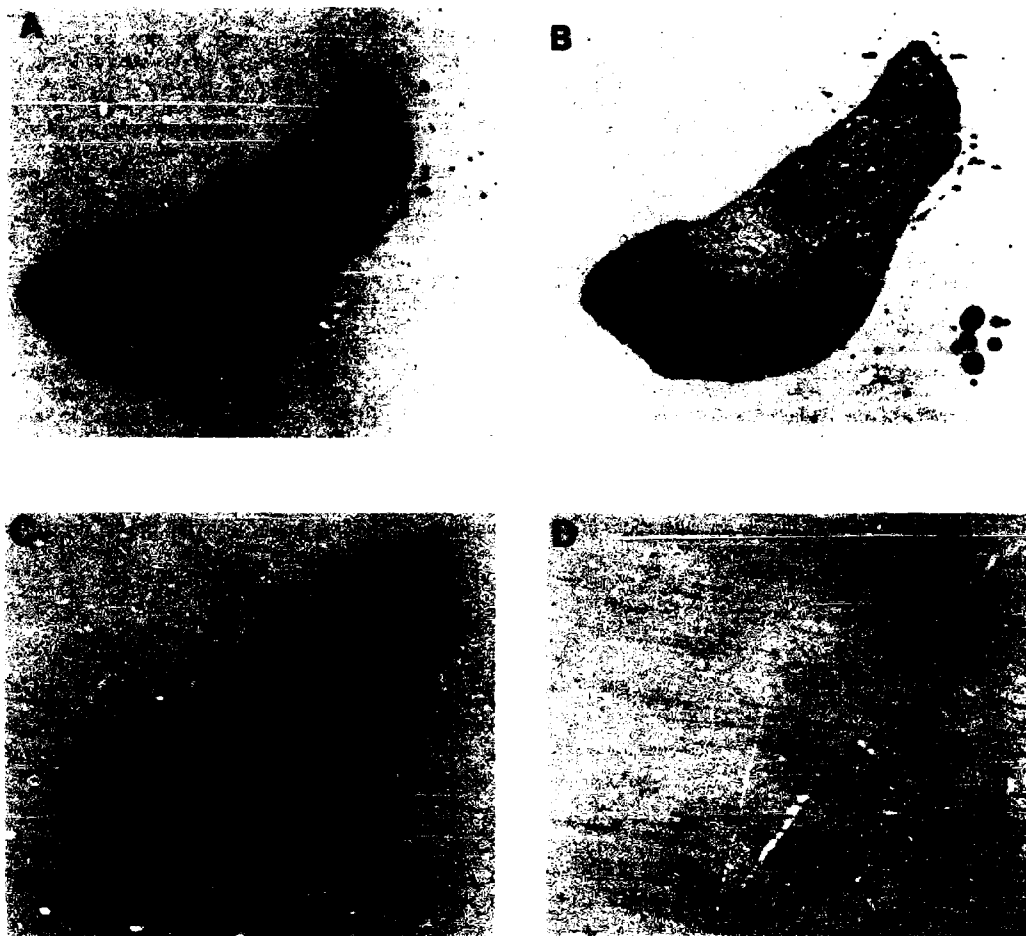


Fig. 1. Autoradiographic localization of [ $^3$ H]PZ and [ $^3$ H](−)QNB binding sites in the human frontal cortex. Post-mortem slide-mounted tissue was pre-incubated for 30 min in modified Krebs buffer and 1  $\mu$ M atropine was used to define specific binding for both ligands. Total (A) and non-specific (B) [ $^3$ H]PZ binding are shown. [ $^3$ H]PZ binding was determined using 20 nM [ $^3$ H]PZ at 25  $^{\circ}$ C for 1 h. Slide-mounted tissue sections were washed twice for 10 min at 0–4  $^{\circ}$ C. Total (C) and non-specific (D) [ $^3$ H](−)QNB binding are shown. [ $^3$ H](−)QNB binding was determined using 1 nM [ $^3$ H](−)QNB at 25  $^{\circ}$ C for 2 h. Slide-mounted tissue sections were washed 3 times for 10 min at 0–4  $^{\circ}$ C. White matter (clear central areas) shows minimal grain density, while the various laminae of the grey matter (dark outer areas) demonstrate high grain density for both ligands.

**Table II.** Reduction in choline acetyltransferase activity and binding of cholinergic ligands to rat anterior cerebral cortical homogenates with bilateral ibotenic acid-induced lesions of nucleus basalis magnocellularis

Parameter	Lesioned (% control)	
	Right	Left
ChAT activity <sup>a</sup>	58.8***	57.5***
[ <sup>3</sup> H](−)QNB <sup>b</sup>	88.5	85.5
[ <sup>3</sup> H]PZ <sup>b</sup>	90.2	81.1*
[ <sup>3</sup> H](+)-CD <sup>b</sup>	73.4*	78.8*
[ <sup>3</sup> H]HC-3 <sup>b</sup>	58.8**	51.9**

<sup>a</sup> ChAT activity (in nmol [<sup>14</sup>C]acetylcholine formed/mg protein/h at 37 °C).<sup>b</sup> Saturation isotherms (*N* = 4) conducted as previously described.<sup>35</sup>\* *P* < 0.05, \*\* *P* < 0.02, \*\*\* *P* < 0.01, as compared to the unlesioned controls.

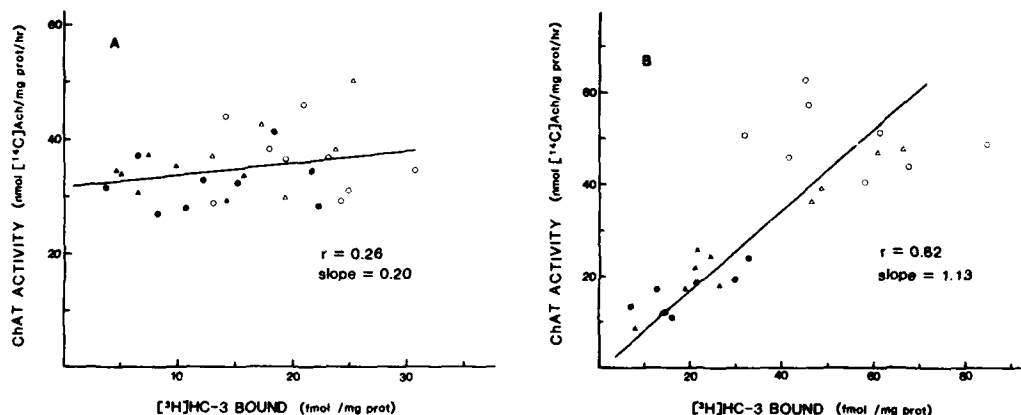
caudate-putamen, nucleus accumbens, olfactory tubercle, amygdala, habenula, and the granular layer of the dentate gyrus. In contrast, extremely few sites were found in the corpus callosum, a white matter region. The distribution of [<sup>3</sup>H]HC-3 binding is compatible with a selective labeling of central cholinergic nerve terminals, and [<sup>3</sup>H]HC-3 binding appears to be of great utility in the identification *in vitro* of sites identical or closely related to the sodium-dependent high affinity choline uptake carrier in cholinergic nerve terminals. Light microscopic autoradiographic localization offers a highly selective method for labeling cholinergic nerve terminals, and for quantitatively assessing the status of these cholinergic nerve terminals throughout the CNS in normal and pathological conditions, as we have been attempting to do in our recent lesion studies.<sup>35,36</sup>

#### Senile dementia of the Alzheimer's type and its animal model

A selective loss of mAChR in the hippocampus and cerebral cortex in Senile Dementia of the Alzheimer's type (SDAT) has previously been reported.<sup>37-39</sup> However, others have had difficulty in finding any alterations in the

mAChR in SDAT. This may be due to selective alterations in mAChR subtypes. In this regard, Mash *et al.*<sup>37</sup> have noted a selective reduction in the M<sub>2</sub> mAChR subtype in SDAT. Significant changes in agonist binding were also seen.<sup>37</sup> We are now examining additional human (Fig. 1) and SDAT brains using quantitative autoradiographic methods to ascertain whether discrete areas within tissues like the hippocampus and cortex show differences in mAChR subpopulations. If the postsynaptic mAChR is functionally intact, this would augur well for the future use of potentially selective mAChR agonists for treating SDAT.

Interestingly, on the basis of our animal studies using bilateral lesions of the nucleus basalis magnocellularis (Meynert in primates), we have speculated that the highest affinity agonist state labeled by [<sup>3</sup>H](+)-CD may be more closely associated with presynaptic nerve terminals, while [<sup>3</sup>H]PZ and [<sup>3</sup>H](−)QNB appear to label predominantly postsynaptic binding sites (Table II)<sup>35</sup> in the rat cerebral cortex. As can also be seen in autoradiographic data (Table I), it appears that while the high affinity binding of [<sup>3</sup>H](+)-CD more closely parallels the binding of [<sup>3</sup>H]HC-3 than it does the binding of either



**Fig. 2.** Correlation analysis of ChAT activity and [<sup>3</sup>H]HC-3 binding in control and AF64-A-treated rats. Assays were conducted as described previously.<sup>36</sup> Paired values of ChAT activity and [<sup>3</sup>H]HC-3 binding in individual rats are shown for the cerebral cortex (A), and hippocampus (B). For vehicle-treated (open) and AF64A-treated rats (filled), the time lapse between intracerebroventricular injection and decapitation was 7 (circles) or 21 (triangles) days. Statistical significance of the regression coefficient (*r*) was determined by *t*-tests. Reprinted by permission.<sup>36</sup>

[ $^3\text{H}$ ]PZ or [ $^3\text{H}$ ](−)QNB, there are some notable exceptions such as in the olfactory tubercle where [ $^3\text{H}$ ](+)[CD binding is relatively low although [ $^3\text{H}$ ]HC-3 binding is high. Thus, whereas [ $^3\text{H}$ ]HC-3 appears to be useful as a highly specific label for presynaptic sites and [ $^3\text{H}$ ]PZ and [ $^3\text{H}$ ](−)QNB appear to label predominantly postsynaptic sites, the interrelationship between each of these highly specific ligands remains intriguing but poorly understood. Perhaps even the close relationship between choline acetyltransferase (ChAT) activity and [ $^3\text{H}$ ]HC-3 binding is not a simple one (Fig. 2).<sup>36</sup>

#### Muscarinic effector systems

In many receptor systems, subtypes have been linked to separate biochemical responses or effector systems. Since muscarinic receptors mediate both the stimulation of phosphatidylinositol (PI) breakdown and the inhibition of adenylate cyclase,<sup>40,41</sup> many investigators have turned to studies of these responses in an effort to identify whether or not there are functional correlates for the putative mAChR subtypes. We have previously speculated that the  $M_1$  mAChR might be coupled to PI turnover while the  $M_2$  mAChR might be coupled to the inhibition of adenylate cyclase.<sup>10,11,15,23</sup>

We, and others, have recently reported that PZ distinguishes putative mAChR subtypes in the rat cerebral cortex based upon the correlation of the high affinity of PZ in studies of PI breakdown and in binding studies.<sup>42,43</sup> It was shown that PZ has a  $pA_2$  value of  $8.4 \pm 0.03$  for the inhibition of CCh stimulated phosphatidic acid synthesis in cholinergically enriched rat synaptosomes.<sup>43</sup> Similar results for PZ have been obtained in rat corpus striatum (Fig. 3). No significant difference could be seen in the antagonism by PZ of the CCh (1.0 mM) stimulated incorporation of [ $^3\text{P}$ ] into synaptosomal phosphatidic acid in these two tissues. There appears to be growing agreement that PI breakdown can be linked to the  $M_1$  mAChR.

The mouse anterior pituitary tumor cell (AtT-20) line has been extensively used for studies of neuroendocrine functions,<sup>44</sup> and mAChRs have been shown to be inversely coupled to adenylate cyclase in these cells.<sup>45</sup> We have recently found that these intact cells, which bind muscarinic antagonists in a manner similar to that seen previously in homogenates of NG108-15 and the rat cerebral cortex, also show mAChR-mediated stimulation of PI turnover (Akiyama *et al.*, submitted). Thus, this cell line offers the opportunity to study both biochemical responses (stimulation of PI turnover and the inhibition of adenylate cyclase) under the same conditions. In these cells PZ showed a 40-fold greater affinity for reversing CCh-stimulated PI turnover ( $K_i = 7$  nM) when compared to PZ antagonism of the inhibition of c-AMP formation ( $K_i = 300$  nM), while atropine was equipotent on both these responses ( $K_i = 0.5$  nM). These values are in good agreement with those previously reported in the rat brain for these two responses.<sup>42</sup> The above studies suggest that PZ distinguishes between mAChR-mediated stimulation of PI turnover and inhibition of adenylate cyclase.

Cardiac mAChRs are inversely coupled to a macromolecular membrane complex ( $N_1$  and  $N_2$  guanine nucleotide binding proteins,  $\beta_1$ -adrenergic receptors and adenylate cyclase) and inhibit the intracellular synthesis of c-AMP. Enzymatically dissociated rat heart cells can

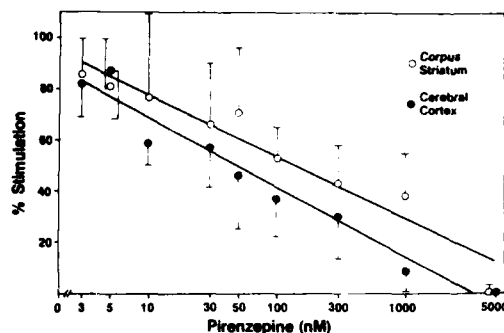


Fig. 3. Antagonism by PZ of the muscarinic agonist-induced stimulation of [ $^{32}\text{P}$ ] incorporation into synaptosomal phosphatidic acid. Carbamylcholine (1 mM) stimulated synthesis in the rat cerebral cortex and corpus striatum are compared. Values represent the means determined from 4–7 separate experiments  $\pm$  S.D. The methods were previously described.<sup>43</sup>

be used to obtain estimates of pharmacological parameters for drugs that selectively interact with the mAChR and inhibit (−)isoproterenol-stimulated c-AMP formation (manuscript in preparation). Elevation of endogenous c-AMP by (−)isoproterenol is rapid (near maximal in 15 s), highly potent ( $EC_{50} = 20$  nM), of great magnitude (11-fold stimulation) and completely reversed by acetylcholine (ACh). Full mAChR agonists (ACh, cis-methyldioxolane, CCh, methacholine, oxotremorine, and oxotremorine-M) and partial agonists (pilocarpine and 4-(*m*-chlorophenylcarbamoyloxy)-2-butylnyltrimethylammonium chloride or McN-A-343) show potent ( $EC_{50} = 70$ –1000 nM) inhibitory effects on (−)isoproterenol-stimulated c-AMP formation. Moreover, CCh (Hill value = 0.54) seems to interact with this receptor-effector complex in a manner different from ACh (Hill value = 0.94). In studies using ACh, atropine ( $K_i = 1$ –2 nM) was 1000-fold more potent than PZ ( $K_i = 1$ –2  $\mu\text{M}$ ) in antagonizing this response. These results suggest that distinct agonist affinity states of the putative  $M_2$  mAChR mediate this c-AMP response in whole cardiac cells and that PZ has low affinity for the c-AMP response in the periphery as well as the CNS.

However, in some systems there is evidence accumulating to suggest that PI breakdown may also be associated with the putative  $M_2$  mAChR.<sup>46,47</sup> Unlike the high affinity values for PZ inhibition of PI breakdown in the rat cerebral cortex and the corpus striatum (Fig. 3), there are differences in the regional affinity values for PZ in the guinea pig.<sup>47</sup> While no differences were found in the capacity for PZ to inhibit [ $^3\text{H}$ ](−)QNB binding, PZ displayed a much lower affinity to alter PI turnover in the guinea-pig neostriatum ( $K_i = 160$  nM) when compared to the cerebral cortex ( $K_i = 12$  nM) and the hippocampus ( $K_i = 14$  nM). Atropine behaved similarly in all three regions. Thus, while species differences and regional differences are becoming apparent, the differences could be accounted for by functionally distinct receptor subtypes or by regional differences in the efficiency of mAChR coupling to inositol phospholipid hydrolysis.

**Table III.** Comparison of  $IC_{50}$  values for muscarinic antagonists in rat heart, brain and human neuroblastoma cell line

	Heart	Cerebellum	Cerebral cortex	SH-SY5Y
Atropine	$2.95 \pm 0.06$	$1.6 \pm 0.1$	$2.3 \pm 0.2$	$1.30 \pm 0.1$
Pirenzepine	$950 \pm 80$	$590 \pm 40$	$159 \pm 9.0$	$120 \pm 20$
AF-DX 116	$104 \pm 3$	$100 \pm 20$	$2400 \pm 100$	$560 \pm 50$

Values (in nM) represent mean  $\pm$  S.D. for each compound. Assays were conducted using 0.23 nM of  $^{125}I$ (-)-QNB at 25°C in modified Krebs phosphate buffer for 2 h ( $N=3$ ) and atropine (1  $\mu$ M) was used to define specific binding.

#### Relationships between muscarinic receptor subpopulations

It is evident that muscarinic agonists bind to mAChR in a manner which differs from muscarinic antagonist binding. Yet it is also apparent that complexities may be found in agonist curves in various functional studies. In addition, these differences between agonists and antagonists appear to be superimposed upon distinct differences between subtypes of mAChR ( $M_1$  and  $M_2$ ) as detected by high affinity PZ binding and the differential regulatory effects of ions, nucleotides and sulphydryl reagents upon high affinity  $[^3H](+)$ CD binding.<sup>10,11,15,23,48</sup>

There exists a great need for selective, competitive  $M_2$  antagonists, and we have recently confirmed (Table III) the findings of Giachetti *et al.* (submitted) and Hammer *et al.* (submitted), which describe the cardioselective properties of AF-DX 116. AF-DX 116, whose chemical name is (11[[2-[(diethylamino) methyl]-1-piperidinyl] acetyl]-5, 11-dihydro-6H-pyrido [2,3-b][1,4]benzodiazepine-6-) one, shows high affinity for the putative cardiac  $M_2$  subtype. Yet, it possesses only low affinity for putative  $M_1$  cerebral cortical mAChR. Interestingly, in the human neuroblastoma cell line (SH-SY5Y) the ligand is also selective (Table III). Both PZ (13 nM, 660 nM) and AF-DX 116 (115 nM, 1300 nM) produce a significant two-site fit in homogenates of SH-SY5Y cells, with 41% and 46% of the sites showing high affinity, respectively. Thus, AF-DX 116 is emerging as an interesting selective  $M_2$  antagonist.

Many of the proposed differences in characteristics of

the putative mAChR subtypes are reviewed in Table IV. These results are based on PZ binding studies and functional studies. They are largely supportive of regionally distinct distributions of mAChR subtypes. In addition to the differences in tissue distribution already discussed, supplementary data supportive of the  $M_1$ ,  $M_2$  concept is outlined. It is becoming more apparent that the regulatory influences of guanine nucleotides, sulphydryl reagents and divalent ions (predominantly  $Mg^{2+}$ ) differ markedly between receptor subtypes. Therefore it should prove useful to elucidate their mechanism of interaction with each putative mAChR subtype since ions and nucleotides could be important endogenous modulators of mAChR function in both the CNS and peripheral nervous systems.

It is also essential that we define the mechanisms by which these receptors are coupled with their respective effectors within the cell membrane. Both putative mAChR subtypes appear to undergo axonal transport. Fig. 4 shows the flow of  $[^3H]PZ$  binding sites. The differential regulation of agonist binding to  $M_1$  and  $M_2$  mAChR by guanine nucleotides and  $Mg^{2+}$ , regulators known to have important roles in other receptor-adenylate cyclase coupled systems, suggests potential differences in the effectors with which  $M_1$  and  $M_2$  mAChR are coupled. Presently, one may argue that differences in drug recognition by  $M_1$  and  $M_2$  mAChRs are the result of different constraints imposed by the effectors with which these putative subtypes are coupled



**Fig. 4.** The autoradiogram shown in (A) depicts the phenomenon of axonal transport of putative  $M_1$  muscarinic receptors in the rat brain. A microelectrode was placed stereotaxically into the brain and a small radiofrequency lesion (arrow) was created in the fimbria (f) of the hippocampal formation. Rats were allowed to survive for 24 h post-lesion before sagittal sections of brains were taken through the lesioned area and prepared for the autoradiographic localization of  $[^3H]PZ$  (20 nM). The tissue was incubated for 1 h at 25°C, as previously reported.<sup>32,34</sup> An accumulation of  $[^3H]PZ$  can be seen surrounding the lesion, indicating that putative  $M_1$  mAChRs are undergoing axonal transport in the fimbria. Non-specific  $[^3H]PZ$  binding is seen in (B). Atropine (1  $\mu$ M) was used in the study to inhibit  $[^3H]PZ$  binding.

Table IV. Proposed characteristics of putative muscarinic receptor subtypes

Characteristic	M <sub>1</sub>	M <sub>2</sub>	References
<b>Tissue distribution</b>			
Cerebral cortex, striatum, hippocampus, ganglia	+++	+	1-15,21,49,47,50
Heart, ileum, pons-medulla, cerebellum, pancreas	+	+++	1-15,17,49,51
<b>High affinity drug binding</b>			
<b>Agonists</b>			
McN-A-343, pilocarpine	+++	++	11,16,17,22,23,48,52,53,58
Carbamylcholine, acetylcholine	++	+++	11,16,17,22,23,48,52,54
Cis-methylcholine, oxotremorine, oxotremorine-M	++	++	11,22,23,48,55
<b>Antagonists</b>			
(-)-QNB	+++	+++	5-15,17,22,23,48
NMS, atropine, scopolamine, dextimide, trihexyphenidyl	+++	++	1-15,17,22,23,48,56
Pirenzepine	+++	+	1-15,17,18,22,23,48
AF-DX 116	+	+++ (?)	this symposium
<b>Regulators of high affinity agonist binding</b>			
Guanine nucleotides	↓	↓↓↓	6-15,17,18,22,23,48
Monovalent cations (Na <sup>+</sup> )	↓↓	↓↓	6,10,11,15,18,22,23,48
Divalent cations (low [Mg <sup>2+</sup> ])	0(?)	↑	6,10,11,15,17,22,23,48
Divalent cations (high [Mg <sup>2+</sup> ])	↓	↓↓	6,10,11,15,17,22,23,48
N-ethylmaleimide, p-chloromercuribenzoate	↑↑	↓↓↓	11,57
<b>Effector systems</b>			
Phosphatidylinositol turnover	+++	+(?)	10,42,43,46,47,59
Adenylate cyclase	+(?)	+++	10,17,18,42
Guanylate cyclase	+++ (?)	0(?)	60
K <sup>+</sup> channel, CNS (M-channel)	+++ (?)	0(?)	
<b>Tissue responses</b>			
Ileum: smooth muscle contraction	0(?)	+++	1
Sympathetic ganglia: excitatory transmission	+++	0(?)	1-3
Heart: contractility, rate	0(?)	+++	3
Gastric acid secretion	+++	0(?)	61-63
CNS-mediated passive avoidance learning	+++	0(?)	64
Pancreatic juice, amylase, insulin release	0(?)	+++	51

(M<sub>1</sub>, PI turnover; M<sub>2</sub>, inversely coupled adenylate cyclase). Indeed, it is possible that within the broad classifications of M<sub>1</sub> and M<sub>2</sub> mAChR, further subclasses of receptor affinity states may also exist as a result of different effector-induced or other membrane-induced constraints.

#### Implications and conclusions

Differences and multiplicity of mAChR's permit fine modulation, and the characterization of multiple mAChR subtypes will aid the development of highly selective efficacious new drugs. However, despite concerns regarding efficacy and the pharmacokinetic concerns which plague *in-vivo*, and even *in-vitro*, studies, it is absolutely necessary to determine the drug response characteristics using pharmacological methods, as well as the ligand binding properties of these sites. Ideally these studies should be done under precisely identical conditions. Moreover, relative potencies of various drugs must show good correlation between binding and pharmacological

data. One should also recall that compounds may interact with ionic channels or secondary allosteric sites which may be associated with the receptor, as well as interacting with the receptor at its primary recognition site.

Further work will no doubt shed light on such issues as the possible contribution of secondary sites, distinct from what may turn out to be one homogeneous primary recognition protein, in producing the relatively high affinity for PZ in the cerebral cortex and the relatively low affinity observed in tissues such as the heart. Ultimately, it is likely that mAChR subclassification will be based upon strict structural criteria. Although there exists some substantial evidence to the contrary,<sup>65</sup> the possibility remains that slight differences such as one single amino acid substitution in the sequence may produce subtle structural differences in recognition sites. [<sup>3</sup>H]PZ may be used to identify M<sub>1</sub> sites, but there is a great need for more selective M<sub>2</sub> drugs. It is even more difficult to define the putative M<sub>2</sub> site or even to say whether or not there may be more than two distinct subtypes. Different

membrane associated proteins or other constituents such as lipids may induce different conformational constraints on receptors in a given tissue. This might facilitate or inhibit the binding to or stimulation of these sites. The existence of different receptor-effector coupling mechanisms remains a possibility, but remains also to be proven. Pirenzepine appears to sense differences in the inactivated states of the mAChR. Coupling to different transducers may itself invoke conformational constraints upon an inactivated state in a manner similar to that previously proposed to explain multiple agonist affinity states in an activated state.

Little doubt exists that selective muscarinic antagonists of great potential therapeutic value are now and will increasingly be made available. Therapeutic and untoward effects of muscarinic drugs may be mediated by independent mAChR subpopulations which may be pharmacologically exploited to produce more highly selective and efficacious new drugs.

An improved understanding of the underlying molecular basis of  $M_1$  and  $M_2$  mAChR subtypes is clearly developing. Certainly the mAChR is now being solubilized, purified and reconstituted, and it will ultimately be sequenced. These studies, and the appropriately cautious interpretation of well designed radioligand binding studies of selective compounds such as [ $^3$ H]PZ, done in parallel with studies of biochemical effectors under the same conditions and/or with biobehavioral studies, represent a promising new probe for the investigation of the etiology and treatment of various disorders. They should provide considerable insight into the etiology and treatment of many insidious CNS disorders such as SDAT, which have already been widely associated with muscarinic dysfunction.

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## Mapping of subtypes of muscarinic receptors in the human brain with receptor autoradiographic techniques

J. M. Palacios, R. Cortés, A. Probst and M. Karobath

*The characteristics and distribution of the putative subtypes of the muscarinic cholinergic receptor were studied in human post-mortem material using receptor autoradiographic techniques.  $^3\text{H}$ -N-methylscopolamine was used as a non-selective ligand and pirenzepine and carbachol were used to displace preferentially the putative  $M_1$  and  $M_2$  sites. In addition,  $^3\text{H}$ -pirenzepine was used to label  $M_1$  sites selectively. Both the selective displacement and the direct labeling resulted in comparable regional distributions of subtypes. Regions enriched in  $M_1$  sites were the basal ganglia, hippocampus, amygdala and substantia nigra.  $M_2$  sites were predominant in the brainstem, cerebellum and thalamus. The cerebral cortex, hypothalamus and basal forebrain contained similar densities of both subtypes. The density and distribution of muscarinic receptor subtypes were examined in selected brain areas from patients dying with Alzheimer's and Parkinson's disease. No changes in the distribution and proportion of  $M_1$  and  $M_2$  receptors were observed in these cases.*

The presence of muscarinic cholinergic receptors (mAChR) in the human brain, presenting pharmacological characteristics similar to those previously described in the laboratory animal is well documented.<sup>1,3</sup> These receptors are altered in a number of neurological and psychiatric diseases.<sup>2,6</sup> Subtypes of mAChR have been described both in the normal and pathological human brain.<sup>2,3,7,8</sup> Application of autoradiographic techniques to the microscopic visualization of mAChR has revealed the association of these sites with specific regions of the brain,<sup>9-13</sup> thus assisting the understanding of the mechanism of action of muscarinic drugs. Using these techniques we have analysed and quantified the distribution of mAChR subtypes in the non-pathological human brain<sup>11,12</sup> and in a limited number of brains from patients who suffered from senile dementia or Parkinson's disease. In this paper we review some of these results.

### Distribution of muscarinic cholinergic receptors in the normal human brain

The characteristics of the binding of  $^3\text{H}$ -N-methylscopolamine ( $^3\text{H}$ -NMS) and  $^3\text{H}$ -pirenzepine ( $^3\text{H}$ -PZ) to human neocortical and striatal tissue sections were analysed biochemically and autoradiographically. Both procedures gave similar results (see below for autoradiographic results): i.e. saturable and high affinity binding for both ligands. Under the conditions used, the affinities ( $K_D$ ) of  $^3\text{H}$ -NMS for mAChR in neocortical and striatal sections were 0.2 and 0.3 nM, respectively. The affinities of  $^3\text{H}$ -PZ, in a Krebs buffer, were 9.0 and 17.0 nM for neocortical and striatal tissue, respectively.

Autoradiography of tissue sections labeled with  $^3\text{H}$ -NMS (Refs 11, 12) revealed a widespread distribution of

mAChR throughout the human brain (Figs 1 and 2). The highest densities were observed in the olfactory tubercle and some basal ganglia including nucleus accumbens, caudate and putamen (Fig. 1), which appeared to have a  $B_{max}$  above 1000 fmol/mg protein. In contrast, very low densities of mAChR were associated with other parts of the basal ganglia, such as globus pallidus (Fig. 1), nucleus subthalamicus and red nucleus. Other structures enriched in mAChR were the amygdala (Fig. 1) and cerebral cortex, where the densities of receptors range from 400 to 600 fmol/mg protein. Similar average concentrations were found in the hippocampal formation, although a differential labeling characterized the distinct layers. Lower densities (200–400 fmol/mg protein) were observed in the diencephalon, basal forebrain (including nucleus basalis of Meynert) (Fig. 1) and septum. In the midbrain (Fig. 2) and brainstem the concentrations of mAChR were in general low; however, we found higher levels of binding associated with specific nuclei including some cranial nerve nuclei like hypoglossus, facialis and trigeminal complex, as well as the solitary nucleus and nucleus paranigralis. The substantia gelatinosa of the spinal cord was also relatively enriched in  $^3\text{H}$ -NMS sites. The lowest densities of mAChR observed correspond to the cerebellum and white matter tracts.

This distribution of mAChR observed in the human brain was analogous to that described, using receptor autoradiography, in the rat brain.<sup>10,13</sup>

### Distribution of muscarinic receptor subtypes

Using quantitative autoradiography we analysed the kinetics of  $^3\text{H}$ -NMS to label mAChR as well as the inhibition of  $^3\text{H}$ -NMS binding produced by carbachol, PZ and atropine in discrete brain nuclei. These studies demonstrated that the proportions of subtypes vary from region to region, although the kinetics of the ligands were comparable in all the regions studied. This is illustrated in Fig. 3 which shows the displacement curves of atropine, PZ and carbachol against  $^3\text{H}$ -NMS in three different brain areas. The dissociation constant ( $K_D$ ) of  $^3\text{H}$ -NMS

Preclinical Research, SANDOZ LTD., CH-4002 Basle, Switzerland.

\*Department of Pathology, Division of Neuropathology, University of Basle, Schönbeinstrasse 40, CH-4003 Basle, Switzerland.

calculated in this way was 0.25 nM, and the inhibition constant ( $K_i$ ) of atropine was 4.3 nM. In the case of carbachol the  $K_i$  was 5.3  $\mu$ M for the high affinity site, and 710.0  $\mu$ M for the low affinity site. The inhibition constants obtained for PZ were 76 nM for the high and 1.4  $\mu$ M for the low affinity site. These values are comparable to those found in binding studies on rat and human brain homogenates,<sup>7,8,14</sup> which indicates that the characteristics of mAChR are not altered in our preparations, and that the autoradiographic approach can be used to evaluate the contents of mAChR subtypes in discrete brain nuclei and areas.

The study of the anatomical distribution of mAChR subtypes has been extended using a single concentration of carbachol or PZ to inhibit <sup>3</sup>H-NMS binding. In addition, we observed that in all areas studied there was a strong correlation between the percentage of  $M_1$  sites and low affinity carbachol sites and vice versa.

Our results indicated that  $M_1$  sites are the main population of mAChR in the basal ganglia (Fig. 1), hippocampus, substantia nigra and layers II–III of the neocortex. Cortical layers IV–VI contained somewhat larger proportions of  $M_2$  sites. The hypothalamus, substantia innominata (Fig. 1) and substantia gelatinosa of the spinal trigeminal nucleus presented similar numbers of  $M_1$  and  $M_2$  sites. Finally, the thalamus, cerebellum and most nuclei of the brainstem contained receptors predominantly of the  $M_2$  subclass. The localization

of  $M_1$  and  $M_2$  receptor populations in the human brain is semiquantitatively illustrated in Fig. 4.

This distribution of mAChR subtypes was confirmed by direct labeling of  $M_1$  sites using <sup>3</sup>H-PZ itself (Fig. 2). Autoradiograms obtained with this ligand show that the striatum, amygdala and hippocampus are heavily labeled by <sup>3</sup>H-PZ while almost no binding was observed in the cerebellum and brainstem.

#### Muscarinic receptors subtypes in Alzheimer's and Parkinson's disease

The deficit of presynaptic cholinergic markers in Alzheimer's disease is a well established feature of this disease. The analysis of mAChR in Alzheimer's brains has however, yielded controversial results with the majority of studies reporting no change in the density of mAChR (see Ref. 15, for a review). These results however, were contested by recent publications.<sup>16,17</sup> Results of the examination of mAChR subtypes in Alzheimer's disease are also controversial, with either no change<sup>7</sup> or a selective loss of  $M_2$  sites<sup>16</sup> being reported.

Receptor autoradiography is well suited for the analysis of pathological tissues because it allows concomitant study of receptor and histopathological changes in the same tissue, with light microscopic resolution. We focused our study first in the hippocampal formation<sup>18,19</sup> because of the predominant involvement of this region in the disease. mAChR were found to be variably affected in



Fig. 1. Autoradiograms from consecutive sections of the human brain labeled with A: 1 nM <sup>3</sup>H-NMS; B: 1 nM <sup>3</sup>H-NMS and 100  $\mu$ M carbachol, the 17% of high affinity agonist sites and the 78% of low affinity sites are visualized; C: 1 nM <sup>3</sup>H-NMS in the presence of 300 nM pirenzepine, the 45% of  $M_1$  sites and the 77% of  $M_2$  sites are still labeled. Observe that carbachol strongly inhibits <sup>3</sup>H-NMS binding to the substantia innominata (SI), while the amygdala and basal ganglia are not affected; in contrast, pirenzepine has more effects in the putamen (Put), globus pallidus pars lateralis (GPI) and lateral (La) and basal (Ba) amygdaloid nuclei. Bar = 5 mm. From Ref. 12.

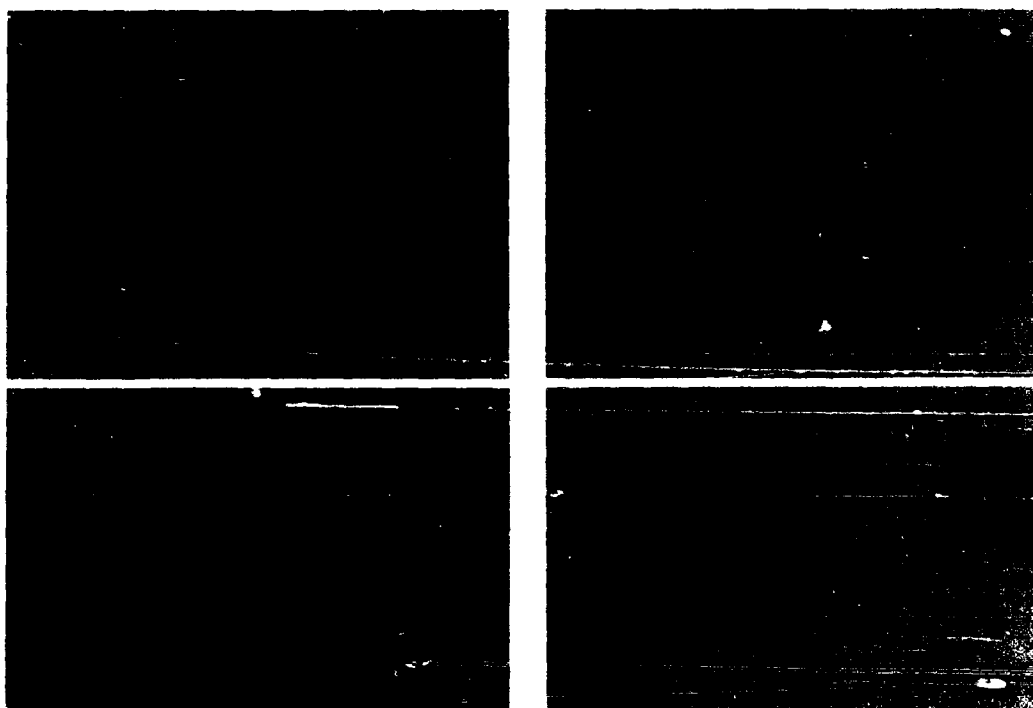


Fig. 2. Autoradiographic images of the human midbrain labeled with A: 1 nM  $^3\text{H}$ -NMS; B: 1 nM  $^3\text{H}$ -NMS and 100  $\mu\text{M}$  carbachol; C: 1 nM  $^3\text{H}$ -NMS and 300 nM pirenzepine; D: 10 nM  $^3\text{H}$ -pirenzepine. Note the blockage of  $^3\text{H}$ -NMS binding to the superior colliculus (SC), griseum centrale mesencephali (CGM) and also pulvinar nucleus of the thalamus (Pu) produced by incubation with carbachol. On the contrary unlabeled pirenzepine inhibits more strongly binding to the cerebral cortex (Cx). The autoradiogram obtained with  $^3\text{H}$ -pirenzepine (D) shows almost no binding in the midbrain and low densities in the thalamus, while the cerebral cortex is densely labeled. Bar = 5 mm. From Ref. 12.

the diseased hippocampi. Cases showing large numbers of senile plaques, the pathological hallmark of the disease, presented normal densities of mAChR, which are predominantly of the  $M_1$  type. Furthermore, the pattern of distribution and the density of receptors was unaffected by the presence of senile plaques. In fact, the neuropil of these plaques appeared, at the anatomical resolution of this technique, as rich in receptors as other neighboring areas without plaques.<sup>18</sup> However, other Alzheimer's cases presented decreased mAChR densities.<sup>19</sup> The characteristics of these cases were (i) severe neuronal loss, (ii) frequent extracellular remnants of neurofibrillary tangles, and (iii) few senile plaques. Receptor loss appeared to be a non-specific effect consequent to neuronal loss since other receptors examined in the same class were also changed and a good correlation between receptor density and neuronal loss was found. These results indicate that mAChR in the hippocampus are located in neuronal elements from intrinsic hippocampal neurons, a fact further confirmed by marked receptor losses observed in hippocampi from non-demented patients presenting localized vascular lesions which result in a well delimited neuronal loss and marked gliosis.

In another series of experiments some cortical areas

from Alzheimer cases were studied. As a control the basal ganglia of these brains were also examined. In cases where a marked loss of serotonin-2 receptors was seen autoradiographically (Pazos *et al.*, in preparation), no change in density, localization or subtype proportions were observed. This confirms previous biochemical studies.<sup>20,21</sup> mAChR in the basal ganglia of these patients were also unchanged.

Another neurodegenerative disease where cholinergic modifications have been reported is Parkinson's disease.<sup>2</sup> We have examined the density and subtype composition of mAChR in the substantia nigra of three Parkinson's brains and the putamen of four cases. Significantly increased densities of mAChR binding were observed in the substantia nigra of the three cases with no change in the putamen. Here again the ratio of  $M_1$  to  $M_2$  subtypes were unaffected by the disease.

#### Summary

Our autoradiographic studies have shown that the putative mAChR subtypes, as defined by the differential affinities of agonists and antagonists, are present in the human brain, where they exhibit a highly heterogeneous distribution. The  $M_1$  subtype is predominant in the

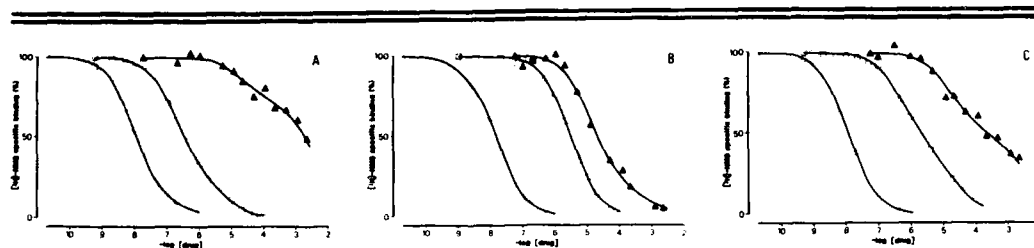


Fig. 3. Displacement curves of 0.5 nM  $^3\text{H}$ -NMS by atropine (O), pirenzepine (□) and carbachol (▲) in three regions of the human brain. A: caudate, B: nucleus nervi facialis and C: substantia innominata, as determined by microdensitometry. From Ref. 12.

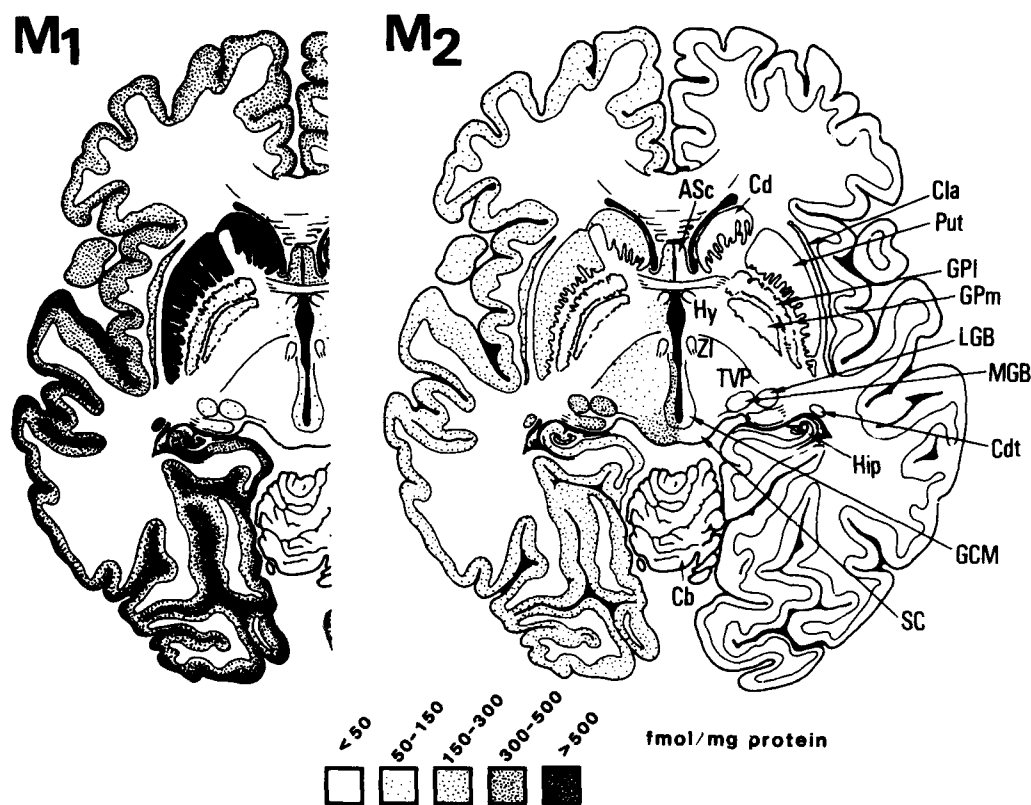


Fig. 4. Distribution of  $M_1$  and  $M_2$  receptor subtypes in the human brain. Receptor concentrations represent  $B_{\text{max}}$  values and were calculated from the selective inhibition of  $^3\text{H}$ -NMS binding by carbachol. Abbreviations: ASc: area subcallosa; Cb: cerebellum; Cd: caudate; Cdt: tail of the caudate; Cla: claustrum; GCM: griseum centrale mesencephali; GPI: globus pallidus pars lateralis; GPm: globus pallidus pars medialis; Hip: hippocampus; Hy: hypothalamus; LGB: lateral geniculate body; MGB: medial geniculate body; Put: putamen; SC: superior colliculus; TVP: nucleus ventralis posterolateralis of the thalamus; ZI: zona incerta.

forebrain, where it is enriched in areas (such as the neocortex and hippocampus) involved in learning and memory processes.  $M_2$  sites are particularly rich in the midbrain and brainstem and could be involved in the cholinergic control of vegetative functions. Furthermore,

these receptors are present in high densities in neocortex and hippocampi from patients who died of Alzheimer's disease. Selective  $M_1$  and  $M_2$  muscarinic agents could thus represent new therapeutic tools in the treatment of neurological diseases, particularly Alzheimer's disease.

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## Inositol lipids and signal transduction at CNS muscarinic receptors

Stephen K. Fisher

*The addition of muscarinic agonists to guinea pig cerebral cortex (brain slices or nerve-ending preparations) results in an increased breakdown of inositol phospholipids, with the concomitant formation of inositol phosphates and diacylglycerol. Individual muscarinic agonists differ in their ability to enhance phosphoinositide breakdown, a property related to the complexity with which the agonist binds to the muscarinic receptor. In guinea pig brain, there are regional differences in the potencies of full agonists, efficacies of partial agonists, and ability of the antagonist pirenzepine to block the response. It is proposed that these differences are due either to regional variations in coupling efficiency of a single muscarinic receptor, or the presence of distinct muscarinic receptor subtypes mediating the phosphoinositide response.*

### Biochemical mechanism

The Hokins<sup>1</sup> were the first to demonstrate that the activation of muscarinic cholinergic receptors (mAChRs) in brain slices resulted in an enhanced turnover of inositol lipids and phosphatidate (PA), as measured by an increase in the incorporation of added  $^{32}\text{P}$  into PA and phosphatidylinositol (PI). This observation has since been confirmed in a variety of neural preparations, including nerve ending preparations, cultured cells of neuronal origin and intact tissues (see Ref. 2 for review). In the central nervous system, the mAChR response is localized predominantly to neurons, rather than glia.<sup>3</sup> The increases in  $^{32}\text{P}$  incorporation are not accompanied by parallel increases in the incorporation of [ $^3\text{H}$ ]-glycerol or [ $^3\text{H}$ ]-glucose, indicating that the  $^{32}\text{P}$  labeling effect is not attributable to a net increase of lipid synthesis, but rather to stimulated turnover of pre-existing lipid moieties. Increased [ $^{32}\text{P}$ ]PA and [ $^{32}\text{P}$ ]PI labeling in the presence of muscarinic agonists stems from an increased availability of diacylglycerol (DAG), derived from the receptor-linked breakdown of a preformed lipid. DAG is phosphorylated in the presence of DAG kinase and the [ $^{32}\text{P}$ ]PA thus formed is subsequently converted to [ $^{32}\text{P}$ ]PI through a cytidine diphospho-diacylglycerol (CDP-DAG) intermediate (Fig. 1). CDP-DAG is presumably also labeled, but is not detected, since it is present in low steady-state amounts. PI may then either be phosphodiesteratically cleaved to DAG, thus completing a PA-PI cycle, or alternatively may undergo a two-stage phosphorylation of the 4' and 5' positions of the inositol ring to yield phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate ( $\text{PIP}_2$ ) respectively. The latter two lipids known collectively as the polyphosphoinositides, are located predominantly in the inner leaflet of the plasma membrane and are especially enriched in neural tissue.<sup>4</sup>

While the source of DAG in ligand-stimulated lipid turnover was long considered to be PI, most current evidence indicates that the initial event following the receptor-ligand interaction in brain is the breakdown of

$\text{PIP}_2$ , which as in other tissues occurs via a phospholipase C route to yield DAG and inositol trisphosphate ( $\text{IP}_3$ ) (Ref. 5). The latter undergoes rapid dephosphorylation to inositol bisphosphate ( $\text{IP}_2$ ) and inositol monophosphate ( $\text{IP}_1$ ), and thence to free inositol which becomes available for the renewed synthesis of the inositol lipids (Fig. 1). The process is metabolically expensive in that for each mol of  $\text{PIP}_2$  that is broken down and resynthesized in response to mAChR activation, 3 mol of ATP and 1 mol of CTP are consumed, a consideration which may point to the physiological importance of inositol lipid turnover in cell function. Under normal circumstances, the release

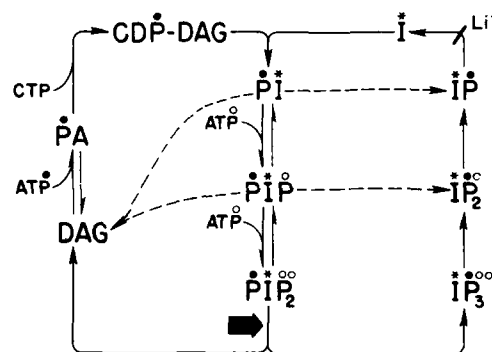


Fig. 1. Cycle of stimulated turnover of inositol lipids.  $^{32}\text{P}$  label ( $\bullet$ ) may enter the cycle in the form of [ $^{32}\text{P}$ ]ATP at the DAG kinase step, resulting in the formation of [ $^{32}\text{P}$ ]PA. Labeled CDP-DAG and PI are subsequently formed and the  $^{32}\text{P}$  label resides in the phosphodiester linkage. Alternatively, the  $^{32}\text{P}$  label may be introduced at the PI and/or PIP kinase steps ( $\circ$ ) at the 4' and 5' positions of the inositol ring respectively. Labeled inositol ( $\text{I}^*$ ) enters the cycle at the CDP-DAG inositol phosphotransferase step, with the resultant formation of labeled PI. The cycle on the right demonstrates the sequential dephosphorylation of  $\text{IP}_3$  to free inositol and inorganic phosphate with an initial loss of the monoester phosphates.  $\text{Li}^+$  ions inhibit the inositol 1-phosphatase.<sup>6</sup> The dotted lines represent possible direct phosphodiesteratic cleavage of PI and PIP. Current evidence favors an initial breakdown of  $\text{PIP}_2$  following the receptor-ligand interaction (indicated by arrow).

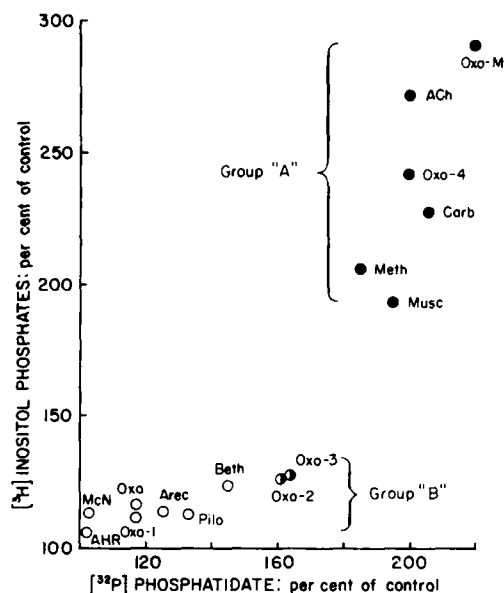


Fig. 2. Relationship between  $[^{32}\text{P}]\text{PA}$  formation in a nerve ending preparation,  $[^3\text{H}]\text{inositol}$  phosphate release from slices, and best fit of receptor occupancy for muscarinic agonists in guinea pig cerebral cortex. Abbreviations used are: Oxo, oxotremorine; Arec, arecoline; Pilo, pilocarpine; Beth, bethanechol; McN, McN-4-343; AHR, AHR-602; Meth, methacholine; Musc, muscarine; Carb, carbamylcholine; ACh, acetylcholine; Oxo-M, oxotremorine-M. For structures of Oxo-1, Oxo-2, Oxo-3, Oxo-4 and Oxo-M, see Ref. 8.  $\circ$ ,  $\bullet$ , receptor occupancy data fits a one- and two-site model respectively.  $\odot$ , receptor occupancy data fits a one- or two-site model.

of the water-soluble inositol phosphates is not readily detected due to their rapid dephosphorylation. However, in the presence of  $\text{Li}^+$  there is an inhibition of inositol 1-phosphatase,<sup>6</sup> resulting in the accumulation of  $\text{IP}_1$ , and to a lesser extent of  $\text{IP}_2$  and  $\text{IP}_3$ . The original observation that  $\text{Li}^+$  can prevent the breakdown of inositol phosphates has been successfully exploited by Berridge and colleagues, and forms the basis of a rapid and specific assay for receptor-linked changes in inositol lipids.<sup>7</sup> Both the increased  $^{32}\text{P}$ -labeling of PA and PI, and the release of labeled inositol phosphates that occur in response to muscarinic agonist addition appear to be dependent upon a minimal availability of calcium ion.<sup>2</sup> However, the phosphoinositide response does not appear to be regulated by the changes in intracellular  $\text{Ca}^{2+}$  concentrations that pertain to physiological stimulation. Rather, the phosphoinositide response likely represents a molecular mechanism whereby cells mobilize cellular calcium.<sup>8</sup>

#### Muscarinic agonist efficacy: relationship to agonist binding characteristics

In the guinea pig cerebral cortex, two groups of muscarinic agonists can be differentiated on the basis of the efficacy with which they enhance the breakdown of

the inositol lipids.<sup>7-9</sup> Addition of optimum concentrations of Group 'A' agonists such as acetylcholine or oxotremorine-M typically elicits a maximum increase in the turnover of inositol lipids, whether measured by the incorporation of  $^{32}\text{P}$  into PA and PI, or by the release of labeled inositol phosphates. In contrast, the addition of Group 'B' agonists such as arecoline or oxotremorine results in an increased turnover of inositol lipids which is only a fraction of that induced by Group A agonists (Fig. 2). Not only are the Group B agonists less effective when added alone, but when present in incubations containing Group A agonists, they block the stimulatory effect of the more efficacious agonists. Thus, Group B agonists typically act as 'partial' agonists—they occupy the same mAChR site as do Group A agonists but are less able to induce the breakdown of inositol lipids. A similar distinction between these two groups of agonists has since been observed for mAChR-enhanced phosphoinositide turnover in rat brain,<sup>10</sup> embryonic chick heart cells<sup>11</sup> and human astrocytoma cells,<sup>12</sup> and in addition, for stimulated cyclic GMP accumulation in murine neuroblastoma NIE-115 cells.<sup>13</sup> In contrast, most Group B agonists are full agonists for mAChR-mediated inhibition of adenylate cyclase.<sup>13</sup>

The ability of a muscarinic agonist to enhance phosphoinositide turnover in the CNS is related to the complexity with which it binds to the mAChR. For example, receptor occupancy curves for Group A agonists obtained from displacement of  $[^3\text{H}]\text{quinuclidinylbenzilate}$  bound to membrane fractions are flattened, with Hill coefficients ( $n_H$ ) of approximately 0.5. These curves can in each instance be resolved into two components, indicating the presence of both high (H) and low (L) affinity forms of the mAChR which differ in  $K_H$  and  $K_L$  (affinity constants) by 30- to 150-fold. In contrast, the receptor occupancy curves for Group B agonists are steeper ( $n_H > 0.85$ ), and can in most instances be adequately described by the interaction of the agonist with a single affinity form of the mAChR, or if not, with two affinity forms of the mAChR which display a minimal  $K_L/K_H$  ratio. This relationship between muscarinic agonist binding characteristics and efficacy for phosphoinositide turnover in the CNS has been observed not only for structurally unrelated compounds (Fig. 2), but also for closely related series of structural analogs of oxotremorine,<sup>14</sup> such as oxotremorine-5, an antagonist, and oxotremorine-M, a full agonist for phosphoinositide turnover (Fig. 3). The results for mAChR-stimulated phosphoinositide turnover are consistent with the model for mAChR activation proposed originally by Birdsall and colleagues,<sup>15</sup> in which differences in agonist efficacy are considered to arise from conformational restraints imposed by the coupling state of the receptor, and agonist efficacy predicted to be a function of  $K_L/K_H$ .

#### Occupancy of the low affinity form of the mAChR evokes inositol lipid breakdown in the cerebral cortex

While full agonists such as oxotremorine-M bind to more than a single affinity form of the mAChR, comparison of dose occupancy curves for the high and low affinity forms of the receptor with the dose response curve for phosphoinositide turnover indicate that it is the low rather than the high affinity form of the receptor that is generally coupled to inositol lipid breakdown (Fig. 4).

Similarly, comparison of the  $EC_{50}$  values for phosphoinositide turnover for the other Group A agonists shown in Fig. 2 reveal that they are in close agreement with the  $K_i$  values obtained from radioligand binding studies.<sup>7,8</sup> These results indicate that Group A agonists are more effective than Group B agonists at recognizing the presence, or inducing the appearance, of the low affinity form of mAChR. Enhanced phosphoinositide turnover appears to result from occupancy of the L-form of the mAChR with little or no receptor reserve for the response. The close relationship between mAChR occupancy and phosphoinositide hydrolysis that exists in the CNS is similar to that observed for the mAChRs in peripheral tissues, and supports the contention that inositol lipid turnover represents a biochemical means of signal amplification at muscarinic receptors.<sup>16</sup> One caveat in this interpretation is that the  $EC_{50}$  values for phosphoinositide turnover are obtained from tissue preparations which possess cellular integrity, whereas agonist binding constants are obtained from membrane preparations. However, the binding constants obtained with membranes and with slice preparations from cerebral cortex are of reasonably similar magnitude. In addition, very similar values for  $K_H$  and  $K_L$  have been obtained for a wide range of muscarinic agonists in whole cells and membranes derived from neuroblastoma N1E-115 cells.<sup>13</sup>

A major unresolved issue is that of the transduction process that intervenes between changes in mAChR conformation and activation of the  $PIP_2$  phosphodiesterase. One possibility that has been considered is the mediation of a guanine nucleotide binding protein, in a manner analogous to that seen with receptors that operate through activation and inhibition of adenylate cyclase. In keeping with this suggestion, the addition of GTP or GppNHP modulates the binding of Group A

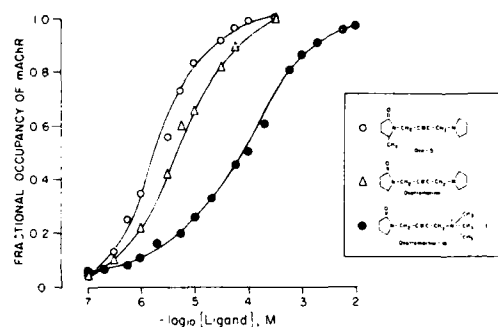


Fig. 3. Occupancy of the mAChR by Oxo-5 (○), oxotremorine (△), and oxotremorine-M (●) as a function of ligand concentration. Displacement of [ $^3H$ ]QNB bound to a nerve-ending fraction by oxotremorine or its analogs was analysed by a computer program which generated values of  $K_H$  and  $K_L$  for a two-site fit and  $K_1$ -site for a one-site fit.<sup>18</sup> Oxo-5 and oxotremorine displacement data fit a one-site model with  $K_1$ -site values of 0.06  $\mu M$  and 0.30  $\mu M$  respectively, whereas for oxotremorine-M, the displacement data is best fitted statistically to a two-site model, with values of  $K_H$  and  $K_L$  of 0.10  $\mu M$  and 13  $\mu M$  respectively. The proportion of high affinity mAChR sites revealed by oxotremorine-M was 24%.

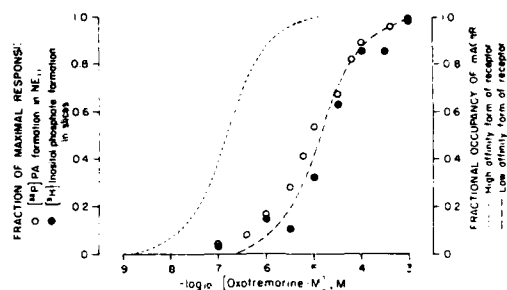


Fig. 4. Concentration dependence of occupancy of the high affinity form of the mAChR (○), low affinity form of the mAChR (●), and stimulation of phosphoinositide turnover for oxotremorine-M. Fractional occupancy of the H- and L-forms of the mAChR was calculated from the equation, occupancy =  $[Oxo-M] / ([Oxo-M] + K_H \text{ or } K_L)$ , where  $[Oxo-M]$  = concentration of oxotremorine-M and  $K_H$  and  $K_L$  are the respective affinity constants (0.15  $\mu M$  and 13.6  $\mu M$ ). ○, [ $^3H$ ]PA formation in a nerve ending preparation ( $EC_{50}$  = 9  $\mu M$ ); ●, [ $^3H$ ]inositol phosphate release from tissue slices ( $EC_{50}$  = 13  $\mu M$ ).

agonists in the guinea pig cerebral cortex (approximately 2-fold shift to lower affinity), whereas there is little or no effect on the binding of Group B agonists. While these results are consistent with the concept that a G-protein may mediate stimulated phosphoinositide turnover in this tissue, further experiments will be necessary to verify this suggestion.

#### Regional differences in coupling mechanisms in guinea pig brain: evidence for muscarinic receptor subtypes or differences in coupling efficiency?

Examination of the characteristics of mAChR-mediated increases in phosphoinositide turnover in three regions of guinea pig brain, cerebral cortex, hippocampus and neostriatum, reveals distinct differences. In the neostriatum, partial agonists such as bethanechol, oxotremorine, or a series of oxotremorine analogs, are markedly more efficacious than they are in either the cerebral cortex or hippocampus. This effect is selective, however, since pilocarpine, arecoline and the putative  $M_1$ -selective agonist, McN-A-343 are equally ineffective in all three regions (Table I).<sup>9</sup> In all three brain regions, the effects of muscarinic agonists on slice preparations appear to be direct since they are unaltered by the inclusion of other agonists or by antagonists which block the effects of other receptor systems linked to phosphoinositide turnover. A second difference between the tissues is that full agonists such as oxotremorine-M and carbamoylcholine are 6- to 29-fold more potent in the neostriatum than in the cerebral cortex, and furthermore, the dose response curves are more shallow.<sup>9</sup> While the latter result might indicate the presence of 'receptor reserve' for the phosphoinositide response in the neostriatum, an alternative explanation is that some or all of the H-receptors are coupled to inositol lipid hydrolysis in this brain region, in addition to the L-receptor. This might also explain the greater efficacy of partial agonists in the neostriatum, since these compounds are presumably able to induce the H-form of the mAChR, even though they

**Table 1.** Effect of partial agonists, full agonists, and antagonists on phosphoinositide turnover in three regions of guinea pig brain.

	Cerebral cortex	Hippocampus	Neostriatum
<b>A. Partial agonists</b> (Efficacy relative to oxotremorine-M)			
Oxotremorine	0.22	0.11	0.40
Oxo-2	0.16	ND	0.61
Oxo-3	0.28	ND	0.63
Bethanechol	0.14	0.16	0.68
Pilocarpine	0.08	0.06	0.06
Arecoline	0.10	0.06	0.25
McN-A-343	0.05	0.06	0.05
<b>B. Full agonists</b> (EC <sub>50</sub> values, $\mu$ M)			
Carbamoylcholine	200	ND	7
Oxotremorine-M	12	ND	2
<b>C. Antagonists</b> (K <sub>i</sub> values, nM)			
Pirenzepine	12	14	160
Atropine	0.4	0.6	0.6

ND = Not determined. Data taken in part from Ref. 9.

ability to induce the appearance of the L-form of the mAChR is limited.

Antagonists also distinguish between the coupling mechanisms in neostriatum and cerebral cortex. Pirenzepine, a putative M<sub>1</sub>-selective antagonist is 18-fold less inhibitory in the neostriatum than in the cerebral cortex, whereas atropine, an antagonist which does not distinguish between the coupling mechanisms is equally effective in both regions. Pirenzepine is also reported to be a weak inhibitor of stimulated inositol lipid hydrolysis in human astrocytoma cells and in embryonic chick heart cells<sup>17</sup> (K<sub>i</sub> = 170–255 nM) and rat parotid gland (K<sub>i</sub> = 124 nM),<sup>18</sup> whereas in rat brain it is a potent inhibitor (K<sub>i</sub> = 10–20 nM).<sup>18</sup> Of additional interest is the observation that pirenzepine binds with equal affinities to the mAChRs in the three regions of guinea pig brain examined. Thus its weak effect on the phosphoinositide response in the neostriatum could not have been predicted. A poor correlation between the binding affinity of pirenzepine obtained from radioligand binding studies and inhibition constants for phosphoinositide turnover has been recently noted for other peripheral tissues,<sup>17,18</sup> indicating a complex mode of interaction of pirenzepine with the mAChR. These studies serve to further emphasize the importance of measuring a functional correlate in addition to radioligand binding studies when characterizing a given population of mAChRs.

While the measurement of inositol lipid hydrolysis clearly indicates differences in the coupling characteristics of the various mAChRs, it would be premature to assign receptor subtypes, i.e. M<sub>1</sub> or M<sub>2</sub> to these responses in the absence of additional pharmacological evaluation. However, if as has been suggested, pirenzepine alone can identify predetermined subclasses of receptors, then it is clear that the phosphoinositide response in guinea pig

brain is mediated by more than one mAChR subtype, a conclusion in agreement with that reached for phosphoinositide responses in rat cerebral cortex and brainstem.<sup>19</sup> An additional complication is that of possible species differences. Phosphoinositide breakdown in rat neostriatum differs from that observed for the guinea pig neostriatum in terms of the response to both full and partial muscarinic agonists (Fisher, S. K., unpublished results). Moreover, pirenzepine is a more potent inhibitor of oxotremorine-M induced inositol phosphate release in rat neostriatum (K<sub>i</sub> = 10 nM v. 160 nM in guinea pig neostriatum). Thus, both regional and species differences may exist in the coupling characteristics of mAChRs in the CNS.

#### Intracellular consequences of inositol lipid hydrolysis and physiological sequelae

The net effect of the mAChR-mediated breakdown of inositol lipids is the production of two important intracellular second messengers, namely inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). There is now general agreement that the production of IP<sub>3</sub> (or perhaps its cyclic derivative?) is intimately linked to the mobilization of cellular calcium.<sup>5</sup> Although direct evidence for this assertion is not yet available in the CNS, initial experiments with cultured human neuroblastoma MCNB-1 cells indicate that muscarinic agonist addition results in an increase in intracellular Ca<sup>2+</sup> (as determined by Quin-2 fluorescence), and that Group A agonists e.g. carbamoylcholine and oxotremorine-M, are more effective than those in Group B, e.g. oxotremorine, a result to be expected were phosphoinositide turnover and Ca<sup>2+</sup> mobilization to be interrelated (Fisher, S. K. and Snider, R. M., unpublished). The other product of PIP<sub>2</sub> hydrolysis, i.e. DAG, is known in some cells to activate protein kinase C, thus providing a link between lipid and protein phosphorylation.<sup>5</sup> The suggestion that DAG production may also be part of an inhibitory feedback mechanism comes from experiments with phorbol esters which are known to mimic the effects of DAG in activating protein kinase C, and also inhibit the agonist-induced release of inositol phosphates in brain.<sup>20</sup>

Evidence to suggest that the observed differences in muscarinic agonist efficacy for phosphoinositide turnover in the CNS is of physiological relevance has been obtained from two independent electrophysiological studies. Lippa *et al.*<sup>21</sup> have found that while the iontophoretic application of both Group A and Group B agonists elicited an increased firing rate of hippocampal pyramidal cells *in vivo*, the response to the Group A agonists rapidly desensitized, whereas the increased firing rate persisted for a longer period in the presence of Group B agonists. Using guinea pig cingulate cortex slices, McCormick and Prince<sup>22</sup> observed that Group A agonists (e.g. oxotremorine-M, muscarine, carbamoylcholine or acetylcholine) were markedly more effective in generating the slow excitatory response associated with an increased input resistance than were the Group B agonists (e.g. oxotremorine, pilocarpine or McN-A-343). In contrast, the rapid inhibitory response elicited by muscarinic agonists in this tissue, and associated with a decreased input resistance, does not appear to be related to the phosphoinositide response.<sup>22</sup> These results suggest that either separate mAChR subtypes mediate the individual

responses, i.e. slow excitation or rapid inhibition, or that there are different effector mechanisms coupled to a single mAChR.

### Conclusions

The hydrolysis of inositol lipids represents an important biochemical mechanism for signal transduction at mAChRs in the CNS, and precedes changes in intracellular calcium and neuronal firing characteristics. The ability to readily measure a functional correlate of mAChR activation in the CNS may prove invaluable in the more rational design of novel muscarinic neuropharmacological agents.

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## Mechanisms of muscarinic excitatory synaptic transmission in ganglia and brain

D. A. Brown, B. H. Gähwiler,\* S. J. Marsh and A. A. Selyanko†

*In rat sympathetic ganglia and hippocampi, stimulation of cholinergic afferents produce similar muscarinic slow depolarizing postsynaptic currents, which are generated through the inhibition of voltage-dependent K<sup>+</sup>-currents. These K<sup>+</sup>-currents serve as braking currents, limiting the output frequency of the innervated neurones. By suppressing these braking currents, cholinergic stimulation may enhance the response of the neurones to high-frequency inputs, subserving an alerting or attention-directing function rather than a direct transmission function.*

Muscarinic excitatory transmission in both sympathetic ganglia and brain show certain common features which distinguish it from the forms of excitatory synaptic transmission mediated through nicotinic receptors or by excitatory amino acids. First the postsynaptic response to even a single shock is delayed by an interval (~100 ms at least) which far exceeds the normal synaptic delay incurred in nicotinic transmission. Also, the response lasts much longer – seconds, as opposed to milliseconds. Second, the primary depolarizing current appears to be generated by a decrease in resting membrane permeability to K<sup>+</sup> ions, rather than through an increased ionic permeability; that is, the transmitter induces the closure of normally-open ionic channels, instead of the opening of normally-shut channels. These features suggest that the function of the muscarinic transmission process may differ radically from that of the nicotinic or amino-acid mediated processes; instead of initiating action potentials, the major role of the muscarinic process may be to facilitate or reinforce ongoing activity, a modifier rather than a trigger function. Some of these points will be amplified with reference to two loci of muscarinic transmission which have been probed using voltage-clamp electrophysiological techniques, the rat superior cervical ganglion and the rat septo-hippocampal system in tissue culture.

### Rat sympathetic ganglion

Fig. 1 illustrates some muscarinic slow synaptic currents recorded from a rat superior cervical ganglion cell under voltage-clamp *in vitro*, under conditions where the fast nicotinic currents are suppressed with curare. These currents are inward, meaning that they would depolarize unclamped cells; they form the currents underlying the previously described slow excitatory synaptic potential of 'slow epsp'<sup>1,2</sup> and may therefore be

termed slow excitatory postsynaptic currents or 'slow epscs'. Two points are worth noting in Fig. 1a. First, the slow epsc lasts for 20 s or so, even after only one or two preganglionic shocks. Second, with increasing preganglionic train length the current increases in amplitude but not in duration: it can, however, be made several times longer by adding neostigmine (see Fig. 2). Fig. 1b illustrates a further feature of the mammalian slow epsc: in contrast to the nicotinic fast epsc,<sup>3</sup> the slow epsc is reduced in amplitude by membrane hyperpolarization, becoming negligible at potentials negative to -60 mV or thereabouts.

**Conductance change** Fig. 2 illustrates another contrast with the fast epsc: the cell input conductance, measured by the amplitude of the additional inward currents

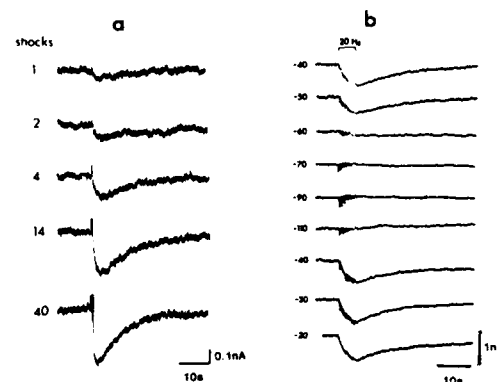


Fig. 1. Slow excitatory postsynaptic currents recorded from two rat superior cervical ganglion cells following repetitive preganglionic nerve stimulation. The ganglia were superfused *in vitro* at 29 °C with a solution containing 400  $\mu$ M d-tubocurarine to block fast (nicotinic) synaptic currents. Cells were voltage clamped through single KCl-filled microelectrodes. Records in (a) show clamp-currents recorded after 1, 2, 4, 14 and 40 preganglionic shocks at 40 Hz at a clamp holding potential of -33 mV. Records in (b) show currents evoked by constant 20 Hz, 5 s trains at different holding potentials. (Adapted from Figs. 1 and 4 in Ref. 10, q.v. for technical details.)

M.R.C. Neuropharmacology Research Group, Department of Pharmacology, University of London School of Pharmacy, 29 39 Brunswick Square, London, WC1N 1AX, UK.

\*Preclinical Research, Sandoz A.G., Basel, CH-4002, Switzerland.

†A.A. Bogomoletz Institute of Physiology, Bogomoltza Str. 4, Kiev-24, USSR.

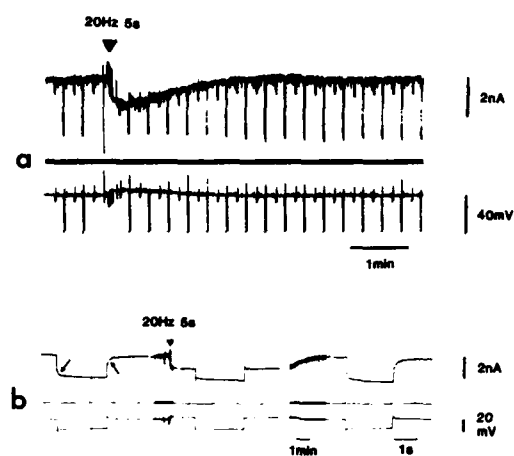


Fig. 2. The ganglionic slow epsc is associated with a fall in input conductance. Records show inward currents following 5 s, 20 Hz preganglionic nerve stimulation recorded in the presence of  $400 \mu\text{M}$  d-tubocurarine and  $1 \mu\text{M}$  neostigmine, with superimposed current deflexions evoked by 2 s hyperpolarizing voltage jumps delivered at 0.05 Hz to measure input conductance. (Upper trace, current; lower trace, voltage). (a) continuous record; (b) high-speed display of currents. Arrows mark inward ( $\downarrow$ ) and outward ( $\uparrow$ ) current relaxations due to voltage-induced deactivation and reactivation of  $I_M$ . (From Ref. 10.)

evoked by constant brief hyperpolarizing voltage commands, is reduced rather than increased during the slow epsc. Inspection of such current trajectories at higher speed (Fig. 2b) shows that this fall in input conductance is associated with an inhibition of the time-dependent current relaxations accompanying the voltage commands. Previous experiments on both amphibian<sup>4,5</sup> and rat<sup>6</sup> ganglion cells have shown that these current relaxations are due to the time-dependent deactivation and reactivation of a species of subthreshold voltage-gated  $\text{K}^+$ -current termed  $I_M$  (M for muscarinic inhibition). We may therefore conclude that the slow epsc in the rat – as in the frog<sup>7</sup> – arises primarily from the inhibition of  $I_M$  and the loss of that component of outward  $\text{K}^+$ -current carried by  $I_M$ . This would explain the voltage-dependence of the slow epsc shown in Fig. 1b: deactivation of  $I_M$  by membrane hyperpolarization reduces and eventually eliminates the source of current driving the slow epsc at a potential ( $-70 \text{ mV}$ ) positive to the  $\text{K}^+$ -equilibrium potential. This precludes the reversal in current direction with membrane hyperpolarization normally expected for a  $\text{K}^+$ -driven process. (In fact, reversal of the slow epsc in mammalian ganglion cells has been detected with membrane hyperpolarization.<sup>8,9</sup> We<sup>10</sup> have also observed reversal of the slow synaptic current in many rat ganglion cells at potentials negative to  $-50/-60 \text{ mV}$ , especially in neostigmine-treated preparations; this we attribute to the parallel inhibition of an outward  $\text{Cl}^-$  current, which is exaggerated in cells impaled with KCl-containing microelectrodes.<sup>11</sup> This effect may contribute little to the inward current driving the slow epsc, but may attenuate the latter under certain circumstances<sup>10</sup>).

**Pharmacology of the slow epsc** The depolarization of the rat isolated superior cervical ganglion by exogenous muscarinic agonists, which probably results in part at least from  $I_M$  inhibition<sup>6,11</sup> (but see also Ref. 12), is readily antagonized by pirenzepine, with a  $\text{pA}_2$  of 8.4 ( $K_i \sim 4 \text{ nM}$ ; Ref. 13). This would accord with an  $M_1$ -mediated system, in agreement with binding data in other ganglia.<sup>14,15</sup> In accordance with this, we find that the slow epsc is substantially reduced in amplitude by  $100 \text{ nM}$  pirenzepine and is virtually annulled at  $1 \mu\text{M}$  (Fig. 3). This block is slowly reversible on washing. Both components of the synaptic response ( $\text{M}$ -current inhibition and the reduced  $\text{Cl}^-$  conductance manifest in the outward current at hyperpolarized potentials<sup>10</sup>) seemed to be equally sensitive to pirenzepine.

**Excitation and excitability changes: the function of the slow epsc** With intense preganglionic stimulation (e.g. in the presence of neostigmine), the depolarization caused by the slow epsc is often sufficiently great to induce sustained trains of spike discharges. However, it is unlikely that the principal function of the slow epsc is the direct transmission of preganglionic spike activity. A more prominent effect, even at subthreshold intensities, is an increased excitability of the ganglion cell; in particular, there is an improved ability to sustain trains of spike discharges when the neurone is challenged with steady suprathreshold current injections (Fig. 4a) and, in extremis, a conversion from phasic to tonic firing behaviour occurs (Fig. 4b). This would accord with previous interpretations of  $I_M$  as an endogenous membrane stabilizing current,<sup>16</sup> a property resulting from its time and voltage-dependent kinetics. Such an effect would

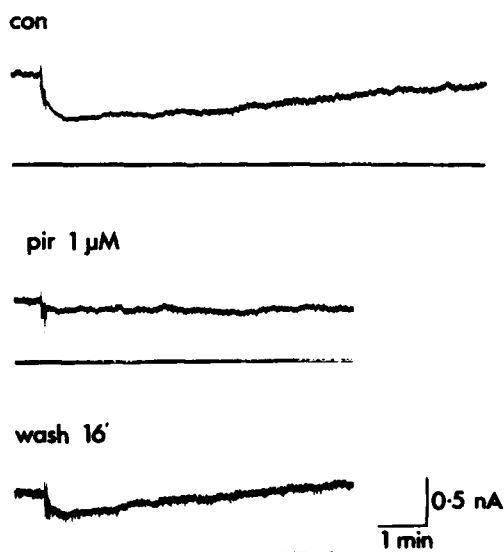


Fig. 3. Pirenzepine (Pir,  $1 \mu\text{M}$ ) reversibly suppresses the slow epsc following 5 s, 20 Hz preganglionic stimulation in  $400 \mu\text{M}$  d-tubocurarine  $1 \mu\text{M}$  neostigmine solution. Holding potential,  $-53 \text{ mV}$ . (Brown, D. A. and Selyanko, A. A., unpublished.)

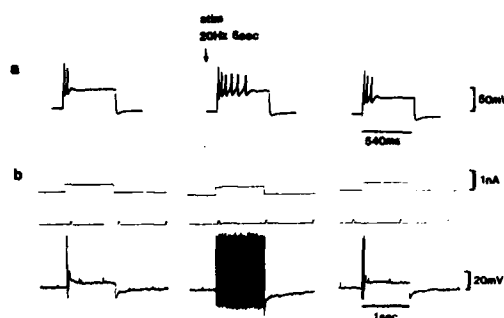


Fig. 4. Facilitation of repetitive firing during the ganglionic slow epsp, recorded (a) in the absence and (b) in the presence of  $1 \mu\text{M}$  neostigmine. (*d*-tubocurarine,  $300 \mu\text{M}$ , was also present in b.) Spikes were evoked by long depolarizing current injections of  $0.8 \text{ nA}$  (a) and  $0.4 \text{ nA}$  (b) while maintaining the membrane potential at a constant (pre-stimulus) level of about  $-50 \text{ mV}$  in each case. Slow epsps were evoked by trains of  $5 \text{ s}$ ,  $20 \text{ Hz}$  preganglionic stimuli. (Record (a), Brown, D. A. and Marsh, S. J., unpublished; record (b), adapted from Ref. 10.)

suggest an appropriate normal function for the muscarinic process to be the facilitation of high-frequency throughput, although direct evidence for this is admittedly weak.

Inhibition of  $I_M$  is not the only mechanism through which repetitive spike discharges can be facilitated. For example, a comparable effect can be induced by inhibiting, with appropriate pharmacological agents, a subclass of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$ -current termed  $I_{\text{AHP}}$  (Ref. 17), in both frog<sup>18,19</sup> and rat<sup>20,21</sup> ganglion cells. Since muscarinic agonists are capable of inhibiting this current in frog ganglion cells<sup>17,22</sup> and in other neurones such as myenteric cells<sup>23</sup> and hippocampal neurones<sup>24</sup> (see below), the question arises how far inhibition of  $I_{\text{AHP}}$  by released acetylcholine (ACh) might contribute to the repetitive firing accompanying the muscarinic slow epsp in the rat ganglion. ( $I_M$  itself is not  $\text{Ca}^{2+}$ -triggered, so  $\text{Ca}^{2+}$ -activated currents do not appear to contribute a steady component of resting membrane current in rat ganglion cell. Thus, inhibition of  $I_{\text{AHP}}$  alone does not induce a membrane depolarization<sup>21</sup> and would not contribute to the inward synaptic current *per se*.) Available evidence suggests a negative answer. Thus, neither exogenous muscarinic nor repetitive preganglionic stimulation reduced the  $\text{Ca}^{2+}$ -dependent spike after-hyperpolarization or the underlying current in rat cells (Fig. 5) under conditions where both were inhibited by noradrenaline<sup>25</sup> or  $\text{Cd}^{2+}$  ions – procedures which block  $\text{Ca}^{2+}$  influx.<sup>26</sup> It appears that the rat ganglion AHP current may be too insensitive to muscarinic receptor activation to permit substantial inhibition by synaptically released ACh.

#### The septo-hippocampal system

The hippocampus receives a strong cholinergic innervation from the medial septum and diagonal band.<sup>27</sup> The principal effect of septal stimulation *in vivo* is to facilitate hippocampal pyramidal cell responses to other non-cholinergic inputs, rather than to elicit directly synchronous pyramidal cell discharges.<sup>28,29</sup> The nature of this

cholinergic inputs, rather than to elicit directly synaptically different experimental approaches. Cole and Nicoll<sup>24,30</sup> stimulated the afferent fibres in the stratum oriens of transverse slices of adult rat hippocampi. In response, they recorded a voltage-dependent depolarization of CA1 pyramidal cells, lasting  $20\text{--}30 \text{ s}$ , which was potentiated by physostigmine and blocked by atropine. This slow epsp was accompanied by (i) an increased cell input resistance; (ii) a burst of action potential discharges if sufficiently intense, and (iii) a facilitation of repetitive firing. The last they attributed to the blockade of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$ -conductances manifest in the characteristically long pyramidal cell spike after-hyperpolarization;<sup>31,32</sup> these after-hyperpolarizations were suppressed by afferent stimulation, and also by the application of

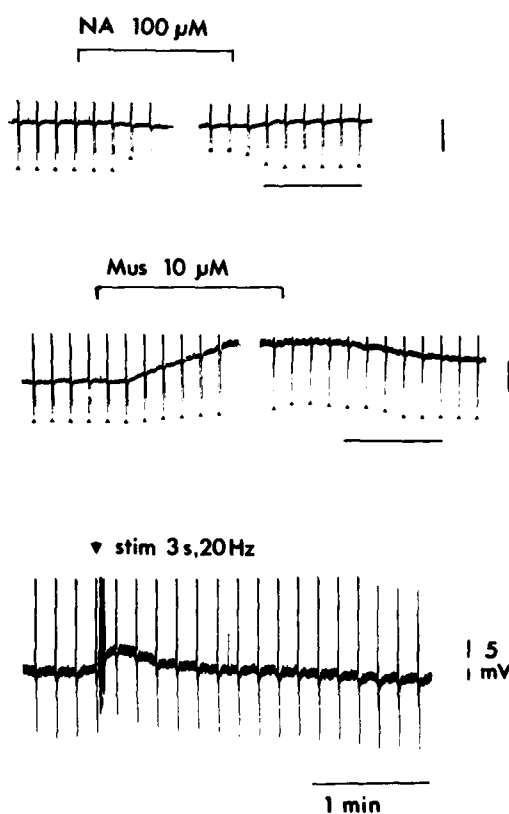


Fig. 5. Effects of (a) noradrenaline ( $\text{NA}$ ,  $100 \mu\text{M}$ ), (b) muscarine ( $\text{mus}$ ,  $10 \mu\text{M}$ ) and (c)  $20 \text{ Hz}$ ,  $3 \text{ s}$  preganglionic stimulation ( $\text{stim}$ ) on spike after-hyperpolarization in the rat superior cervical ganglion. Single spikes were evoked by short ( $5 \text{ ms}$ ) depolarizing current injections. The after-hyperpolarizations, lasting about  $200 \text{ ms}$ , result from activation of a  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$ -conductance by  $\text{Ca}^{2+}$  entry.<sup>36</sup> Noradrenaline inhibits the after-hyperpolarization,<sup>25</sup> probably by reducing the  $\text{Ca}^{2+}$ -current,<sup>26</sup> but does not depolarize the cell. In contrast, neither muscarine nor preganglionic stimulation reduced the after-hyperpolarization; instead, the peak hyperpolarization is increased, probably because of the increased driving force and input resistance. (Brown, D. A. and Marsh, S. J., unpublished.)

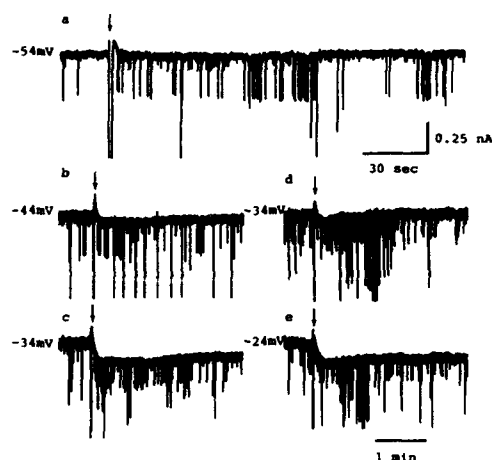


Fig. 6. Voltage-dependence of the cholinergic slow EPSC recorded from a voltage-clamped CA3 pyramidal cell in a cultured hippocampal slice following repetitive stimulation of a co-cultured medial septal slice at 40 Hz for 1 s (at arrows). Between c and d the  $Mg^{2+}$  concentration in the bathing fluid was raised from 4 to 8 mM. Sharp downward deflections are spontaneous fast EPSCs. (Gähwiler, B. H. and Brown, D. A., unpublished; see Ref. 37 for technical details.)

ACh,<sup>24,33-35</sup> histamine<sup>35</sup> and  $Cd^{2+}$  ions,<sup>34</sup> with similar effects on repetitive firing. The synaptic depolarization was, however, unique to the cholinergic system and so could not be attributed to the blockade of the  $Ca^{2+}$ -activated  $K^{+}$ -current. Instead, it might have resulted from inhibition of the voltage-dependent  $K^{+}$ -current,  $I_M$ , in an analogous manner to that seen in ganglia; the presence of an  $M$ -current, susceptible to inhibition by exogenous muscarinic agonists, has been described in voltage-clamped hippocampal pyramidal neurones by Halliwell and Adams.<sup>36</sup>

We<sup>37</sup> have adopted a different strategy for studying the septo-hippocampal system *in vitro*, by co-culturing slices of medial septum and hippocampus from baby rats in close proximity on cover slips.<sup>36</sup> After 3-5 weeks in culture a dense septo-hippocampal innervation develops. It is then possible to voltage-clamp the hippocampal pyramidal cells and to record the evoked synaptic currents produced by stimulating septal neurones. In agreement with recent histochemical findings in adult rats,<sup>39</sup> we detected both cholinergic and non-cholinergic components of functional septo-hippocampal innervation. In CA3 cells, the non-cholinergic component yielded a short-latency, fast (50 ms) inward synaptic current, usually followed by outward currents probably due to activation of GABA-releasing interneurons in the hippocampus. The cholinergic component, detectable in about half of the cultures, and identifiable histochemically by cholinesterase staining, comprised a delayed, low-amplitude and very slow (> 1 min) inward current which was enhanced by neostigmine and blocked by 0.1-1  $\mu$ M atropine. In cells not voltage-clamped, the resultant slow depolarization sometimes induced the burst-firing characteristic of CA3 neurones. In agreement with observations

on adult CA1 neurones,<sup>30</sup> the cholinergic slow EPSC was voltage-dependent, decreasing in amplitude with membrane hyperpolarization (Fig. 6). Current deflections produced by short voltage jumps were reduced during the slow EPSC, indicating a fall in cell input conductance (Fig. 7). As in rat ganglion cells, this fall in conductance seemed to stem largely from a reduction of the time-dependent current relaxations induced by the voltage jumps. Other tests suggested that these relaxations may have reflected de-activation and re-activation of the  $M$ -current previously described in adult hippocampal neurones,<sup>36</sup> since they were resistant to changes in external  $Ca^{2+}$  or addition of  $Ca^{2+}$ -channel blockers, but were inhibited by muscarine or  $Ba^{2+}$  ions.<sup>37</sup> Also in agreement with Cole and Nicoll,<sup>24,30</sup> we noted a reduction in the amplitude of the post-spike hyperpolarizations following septal stimulation, although this was difficult to follow systematically in unclamped cells in the presence of the pronounced burst-firing. For the same reason, we have not been able to ascertain the effects of septal stimulation on repetitive firing behaviour in these cells.

It therefore appears that, in the hippocampus, septal cholinergic stimulation can inhibit two forms of  $K^{+}$ -current. Inhibition of a  $Ca^{2+}$ -activated  $K^{+}$ -current abbreviates post-spike hyperpolarization and facilitates repetitive firing, without producing substantial membrane depolarization; inhibition of a  $Ca^{2+}$ -independent current (probably  $I_M$ ) leads to voltage-dependent slow synaptic depolarization and burst-firing behaviour, and may further facilitate repetitive firing.<sup>40</sup> A pertinent question is whether these two effects might be mediated through different muscarinic receptor transduction mechanisms. Multiple receptors have been detected in hippocampal homogenates by pirenzepine binding assays<sup>41</sup> and two transduction mechanisms for muscarinic receptor activation - accelerated phosphatidylinositol (PI) breakdown and adenylate cyclase inhibition - associated with the higher and lower affinity pirenzepine binding sites have recently been described in rat brain.<sup>42</sup> Phorbol esters have recently been shown to block the  $Ca^{2+}$ -dependent post-spike hyperpolarization in hippocampal cells,<sup>43</sup> leading to the suggestion that muscarinic agonists may accelerate PI turnover, release diacylglycerol and activate C kinase. However, there are a number of caveats to the interpretation of this effect. First, the hyperpolarizing current is

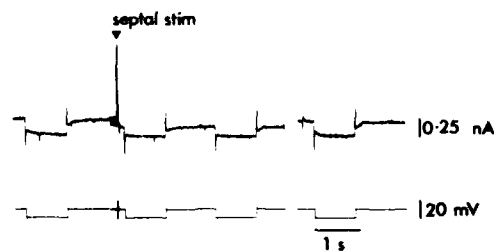


Fig. 7. Fall in input conductance during the slow septo-hippocampal EPSC. Records show inward current deflections produced by 10 mV 1 s hyperpolarizing voltage steps from a holding potential of -40 mV before, immediately after and several minutes after 40 Hz, 1 s septal stimulation. Compare with Fig. 2. (Gähwiler, B. H. and Brown, D. A., unpublished.)

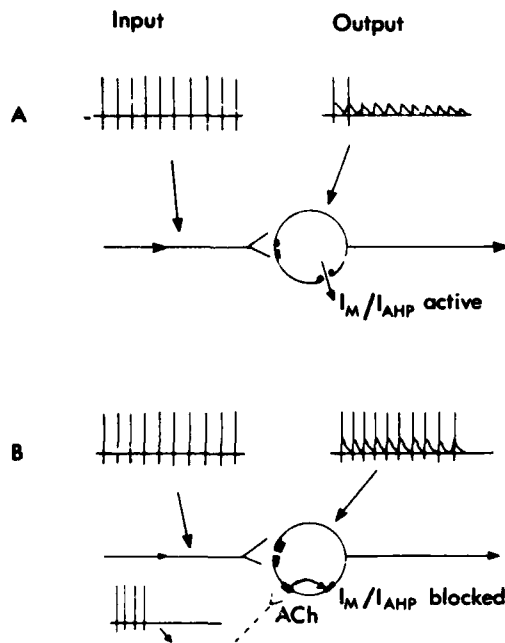


Fig. 8. Hypothetical schema to show how braking currents such as  $I_M$  or  $I_{AHP}$  might limit the output frequency of a neurone (A) and how this limitation might be relieved when these currents are inhibited by cholinergic stimulation (B). See text for details.

also inhibited by other putative 'second messengers', including cyclic AMP (Ref. 34) and cyclic GMP (Ref. 44). Second, it has not yet been established that this component of cholinergic action is mediated through the high-affinity pirenzepine ( $M_1$ ) site; on the contrary, in isolated olfactory cortical neurones the depression of the corresponding  $Ca^{2+}$ -dependent current by muscarinic agonists<sup>45</sup> appears to be relatively insensitive to pirenzepine but sensitive to 10  $\mu M$  gallamine, suggestive of an ' $M_2$ ' system (Sim, J. A. and Constanti, A., unpublished observations). Finally, the second messenger for the inhibition of the  $Ca^{2+}$ -independent M-current system, which appears more closely linked to the  $M_1$ -receptor, remains elusive; available evidence suggests that this is not sensitive to phorbol esters, either in the hippocampus<sup>43</sup> (Nicoll, R. A., personal communication) or in the rat ganglion (Brown, D. A. and Selyanko, A. A., unpublished observations).

### Conclusions

An interesting feature in common to the muscarinic excitatory synaptic systems in ganglia and brain is that the prime targets for transmitter action are  $K^+$ -currents whose normal functions are to stabilize the neurone and limit the output firing frequency. By removing this 'braking' influence, cholinergic stimulation improves the output performance of the neurone when challenged with a depolarizing current injection. Fig. 8 depicts one way in which we may imagine such a system to operate. Under

normal conditions (Fig. 8A), with its braking currents intact (be they  $I_M$  or  $I_{AHP}$  or both) the neurone may be unable to sustain a high frequency output in response to tonic input activity; the output is heavily damped. However, during concurrent cholinergic muscarinic activation, the braking currents are inhibited (Fig. 8B), and the output frequency in response to the same input level may be greatly enhanced. Although highly conjectural, this mechanistic scheme fits in well with the concept of the cholinergic system as an alerting or attention-directing system. Notwithstanding the ability of intense cholinergic activation to directly initiate spike barrages through its depolarizing effect, it seems likely that this facilitatory effect is the most significant feature of cholinergic transmission, particularly in the hippocampus and cerebral cortex. If, as it seems plausible, the control of the different braking currents is mediated through different receptor/transduction mechanisms, then it might prove possible to subtly vary the degree of cholinergic activation by pharmacological means.

### Acknowledgement

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## Pirenzepine discriminates among ionic responses to acetylcholine in guinea-pig cerebral cortex and reticular nucleus of thalamus

David A. McCormick and David A. Prince

*Applications of acetylcholine (ACh) to pyramidal neurones of the cerebral cortex result in inhibition followed by slow excitation. The slow excitatory response appears to be due to a direct decrease in voltage dependent  $K^+$  conductances, while the inhibitory response is indirectly caused by the relatively rapid excitation of inhibitory interneurons which release  $\gamma$ -amino-butyric acid (GABA). Both of these responses are mediated by muscarinic receptors; however, they are pharmacologically distinct with the slow excitation of pyramidal neurones being selectively blocked by low doses of pirenzepine. In contrast to these effects on cortical pyramidal cells and interneurons, ACh causes an increase in membrane  $K^+$  conductance in neurones of the reticular nucleus of the thalamus. The resulting hyperpolarization can interact with intrinsic membrane properties of these neurones to shift the firing mode of the affected neurone from single action potentials to bursts. This response is readily blocked by the muscarinic antagonist scopolamine, but is not easily blocked with pirenzepine. Acetylcholine, therefore, can cause at least three different muscarinic postsynaptic actions (slow excitation, rapid excitation, and direct inhibition) in the mammalian CNS. These responses to ACh occur on different classes of neurones, may be mediated by at least two pharmacologically distinct subclasses of muscarinic receptors, and result from different types of alteration in ionic conductances.*

Interaction of acetylcholine (ACh) with postsynaptic muscarinic receptors in the CNS results in a number of effects including: inhibition manifest by decreases in spike activity and responses to excitatory inputs<sup>1-5</sup>; slow excitation associated with an increase in spike activity and/or an increase in responsiveness to excitatory inputs<sup>1,2,4-7</sup>; and inhibition of single spike activity, paradoxically associated with an enhancement of responses to excitatory inputs.<sup>8,9</sup> Slow excitation in response to ACh has been observed at all levels of the nervous system and appears to be due in large part to the inactivation of both a specialized voltage dependent  $K^+$  current (M-current)<sup>10-13</sup> and another distinct  $K^+$  current which is activated by the entry of  $Ca^{2+}$  ions.<sup>14,15</sup> Both the M-current and  $Ca^{2+}$ -dependent  $K^+$  current are activated by membrane depolarization, exert a dampening or hyperpolarizing influence upon the membrane potential, and are important in the regulation of neuronal spike activities.<sup>16</sup> Muscarinic suppression of these currents therefore removes these 'inhibitory' influences and results in excitation or increased responsiveness to excitatory inputs.

Acetylcholine-induced inhibition of spike activity has also been observed at all levels of the nervous system.<sup>5</sup> However, in the thalamus, the cholinergic 'inhibition' is associated with an enhancement, and not a decrement, in responses to excitatory inputs.<sup>8,9</sup> The mechanisms of ACh-induced inhibition have been investigated only within the peripheral ganglia, where it is mediated by a slow increase in  $K^+$  conductance due to activation of

muscarinic receptors directly on the cell studied,<sup>1</sup> or perhaps through the excitation of adrenergic interneurons.<sup>17</sup>

Numerous pharmacological studies have indicated that muscarinic receptors may be grouped into subclasses according to the binding affinities and actions of various agonists (e.g. oxotremorine, carbachol, pilocarpine) and antagonists (e.g. pirenzepine, gallamine).<sup>18-22</sup> It is entirely possible that the different actions of ACh on central neurones outlined above are mediated by different subclasses of muscarinic receptors. We have recently used the *in-vitro* brain slice technique in conjunction with intracellular recordings to elucidate the mechanisms of action of ACh within the mammalian CNS and to test the hypothesis that the receptor-effector complexes mediating different physiological responses are pharmacologically distinct.

### Actions of ACh in the cerebral cortex

The principal projecting neurone of the cerebral cortex is the pyramidal cell; non-pyramidal cells represent a more heterogeneous group which includes excitatory and inhibitory interneurons. We have tested the effects of ACh on pyramidal neurones in three cortical regions (anterior cingulate, sensorimotor, visual) and have found that ACh has similar actions on these neurones irrespective of location.<sup>23</sup> In brief, application of ACh to pyramidal cells at membrane potentials depolarized to approximately -65 mV results in a short latency inhibition (Fig. 1B, i) followed by a slow and prolonged excitation (Fig. 1B, e). In many neurones, the inhibitory response is concomitant with a barrage of inhibitory postsynaptic potentials (i.p.s.p.'s). In contrast, application of ACh to pyramidal neurones at more negative mem-

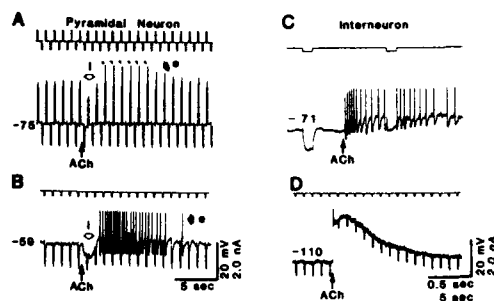


Fig. 1. The effects of acetylcholine (ACh) on physiologically identified pyramidal cells and interneurons. A. Application of ACh to a typical pyramidal cell at resting membrane potential ( $V_m = -75$  mV) initially caused a decrease in the response to the current pulses (i) followed by a selective potentiation of the depolarizing responses without affecting resting  $V_m$  or the response to the hyperpolarizing pulses (e). The potentiated depolarizing responses reached firing threshold and evoked action potentials (downward arrow heads). B. Application of ACh to the neurone of A after depolarization to near firing threshold ( $-59$  mV) caused inhibition at a short latency (i) and was followed by a slow depolarization and action potential generation (e). C. Application of ACh to a typical interneurone at resting  $V_m$  ( $-71$  mV) caused robust excitation at short latency. D. Application of ACh to the interneurone of C after hyperpolarization to  $-110$  mV evoked a large depolarization with a short onset latency. Comparison of responses to hyperpolarizing pulses during ACh-induced depolarization with responses during equivalent direct depolarizations (not shown) revealed that ACh elicited a substantial decrease in input resistance. The top trace in each set (A–D) is the current monitor. In this and all subsequent figures, the intracellular current pulses are 120 ms in duration and were applied at one Hz. Action potential amplitudes are truncated in this and subsequent figures due to the limited frequency response of the inkwriter. Time calibration is 0.5 s for C and 5 s for A, B and D. From McCormick and Prince, Ref. 24.

brane potentials (e.g.  $-75$  mV) results in an initial inhibition (Fig. 1A, i) followed by an increase in responsiveness to depolarizing current pulses, often without substantial change in either resting membrane potential or the response of the cell to hyperpolarizing current pulses (Fig. 1A, e). Thus, ACh causes two major effects on cortical pyramidal neurones: inhibition of short latency followed by a voltage dependent slow depolarization accompanied by increased responsiveness to depolarizing current pulses.<sup>23,24</sup> In addition, we have found that ACh suppresses the slow afterhyperpolarization (a.h.p.) in cortical pyramidal neurones,<sup>23</sup> presumably by blocking a  $Ca^{2+}$  activated  $K^+$  current<sup>25</sup> as is the case in hippocampal<sup>17</sup> and other<sup>14,15</sup> neurones.

The ACh-induced slow depolarization of pyramidal neurones appears to be a direct action (not mediated through the release of other neurotransmitters) since blockade of synaptic transmission with solution containing  $Mn^{2+}$  and low  $Ca^{2+}$ , or with the  $Na^+$  channel blocker tetrodotoxin, fails to abolish this effect.<sup>23</sup> In contrast, both of these manipulations, as well as application of antagonists of GABAergic synaptic transmission (bicuculline, picrotoxin), completely block the

ACh induced inhibition.<sup>23</sup> These data suggest that the slow excitation and suppression of the a.h.p. are direct effects of ACh on pyramidal neurones while the inhibitory response is mediated through the release of the inhibitory neurotransmitter GABA. The slow depolarizing responses elicited by ACh after blockade of synaptic transmission are associated with a voltage-dependent decrease in apparent membrane conductance,<sup>23</sup> which is probably due to decreased  $K^+$  currents (i.e. M-current,  $Ca^{2+}$ -activated  $K^+$ -current).

The ACh-induced inhibition appears to be due to a rapid excitation of GABAergic interneurons. In support of this conclusion, we found that application of ACh to physiologically identified interneurons<sup>26</sup> results in some cases in a rapid excitation (Fig. 1C and D) which is associated with an increase in apparent input conductance.<sup>23,24</sup> Furthermore, extracellular unit recordings have confirmed the presence of a class of cortical neurones which respond relatively quickly to ACh (Ref. 23). Blockade of synaptic transmission (as above) did not eliminate the rapid response to ACh, indicating that the latter is a direct effect on the neurones from which activity was recorded (Fig. 2). Local application of scopolamine completely blocked the excitation by ACh, confirming its muscarinic characteristic (see below and Fig. 2B).

#### Pharmacology of cortical cholinergic responses

The use of *in-vitro* slices maintained in a fluid gas interface type chamber provides a number of advantages to the neurophysiologist including the ease of obtaining stable intracellular recordings. However, one disadvantage of this approach is that the time required for diffusion of pharmacological agents into and out of the brain tissue from the perfusion solution is rather long (equilibrium in 30–60 min). Therefore, in general it is not possible to obtain quantitative dose-response curves for cholinergic agents while maintaining an intracellular recording from a single neurone. In spite of this, we

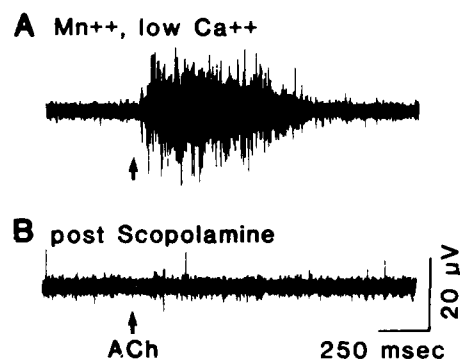


Fig. 2. Example of neurones recorded extracellularly which respond to ACh with a rapid excitation. A. After blockade of synaptic transmission by bathing the slices in solution containing 2 mM  $Mn^{2+}$  and 0.5 mM  $Ca^{2+}$ , ACh still caused rapid excitation of some neurones indicating that this effect is direct and is not due to an ACh-induced release of another excitatory neurotransmitter. B. Local application of a small amount of scopolamine ( $10 \mu M$ ) to the surface of the slice completely blocked the response to ACh.

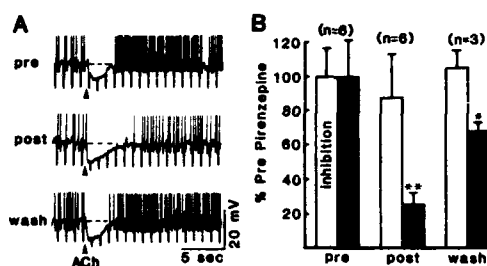


Fig. 3. Effects of  $10 \mu\text{M}$  pirenzepine upon the inhibitory and slow excitatory responses to ACh. A. Application of ACh (upward arrow head) to this neurone at  $V_m$  just above firing threshold evokes the typical inhibitory, slow excitatory response (pre). After approximately  $1 \frac{1}{2}$  exposure to  $10 \mu\text{M}$  pirenzepine ACh application to the same neurone causes the inhibitory response only (post). Washing out the pirenzepine for approximately one hour partially reinstates the slow excitatory response (wash). B. Average data for six neurones showing that  $10 \mu\text{M}$  pirenzepine causes a significant (\*\*,  $p < 0.001$ ) depression of the slow excitatory response without affecting the amplitude of the inhibitory response. When compared to post-pirenzepine responses, washing out the pirenzepine for approximately one hour caused a significant (\*,  $p < 0.05$ ) increase in the slow excitatory response (wash). Data are expressed as percent pre-pirenzepine. Inhibitory responses were measured in mV-ms, and slow excitatory responses as the increase in firing frequency over baseline for the first five seconds of the depolarizing response. From McCormick and Prince, Ref. 24.

have obtained pharmacological data which suggest that the actions of ACh on pyramidal and non-pyramidal neurones are mediated by pharmacologically distinct subclasses of muscarinic receptor-effector complexes.

All responses of cortical pyramidal neurones to applications of ACh (including inhibition and slow excitation) are blocked by the muscarinic antagonists atropine and scopolamine, but not by the nicotinic antagonists dihydro- $\beta$ -erythroidine or hexamethonium<sup>24</sup> indicating that the responses discussed here are mediated by muscarinic receptors. We tested the possibility that the cholinergic inhibition (rapid release of GABA) and slow excitation are mediated through pharmacologically distinct receptor-effector complexes by examining their relative sensitivities to various cholinergic agonists and antagonists. Both responses were blocked by the same threshold dose of the muscarinic antagonist atropine ( $1 \mu\text{M}$ ). In contrast, bath perfusions of pirenzepine (PZ)<sup>19,22</sup> ( $1$  or  $10 \mu\text{M}$ ), preferentially blocked the slow excitatory response on pyramidal neurones, but left the inhibitory response completely intact (Fig. 3). Larger doses of PZ ( $50 \mu\text{M}$ ) completely abolished the slow excitation, but also suppressed the inhibitory response. These antagonist concentrations were those in the superfusing solution; with the interface slice used in these studies, the concentration in the tissue may be less. Some cholinergic agonists varied in their ability to evoke either the slow excitatory, or rapid inhibitory response of pyramidal cells, even though ACh elicited both effects when applied to the same neurones. Applications of carbachol, acetyl- $\beta$ -methacholine, propionylcholine, and

oxotremorine-M were all effective in eliciting both the hyperpolarizing and the slow voltage-dependent depolarizing response. In contrast, pilocarpine and dicholine suberate (both of which differentiate between subtypes of muscarinic responses in the peripheral nervous system<sup>27,28</sup>) and  $\text{Dl-muscarine}$  were all much more effective in eliciting the slow depolarizing than the inhibitory response (Fig. 4), although the slow excitations elicited by pilocarpine were often relatively weak. Applications of oxotremorine, on the other hand, evoked the inhibitory response only (Fig. 4D). Interestingly, further applications of ACh (after oxotremorine) failed to elicit the slow depolarizing response, indicating that oxotremorine may be an agonist at the receptor site mediating the release of GABA and only a partial agonist at muscarinic receptors mediating the slow depolarization of pyramidal neurones.

Applications of the ganglionic stimulant McN-A-343 caused only very weak inhibition and slow excitation in pyramidal neurones; these results agree with some pharmacological data suggesting that this agent is a partial agonist in the cerebral cortex.<sup>29</sup>

Pharmacological studies have shown that muscarinic agonists and some antagonists (e.g. pirenzepine) bind to receptors in a manner which is best explained by the presence of three different subclasses of receptors. These have been termed SH (super-high), H (high) and L (low) agonist affinity sites in the cases of muscarinic agonists;<sup>18</sup> and A (highest affinity), B (low affinity) and C (lowest affinity) sites in the case of pirenzepine.<sup>21,22</sup> In some circumstances, the L agonist sites appear to correspond to

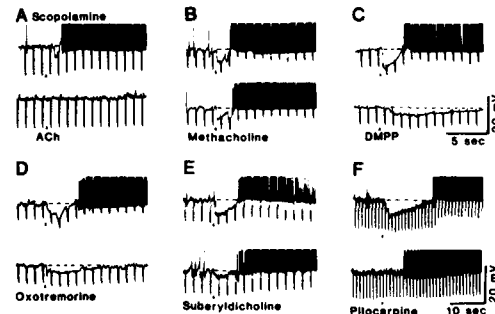


Fig. 4. Comparison of responses to various cholinergic agents with those to ACh. In all sets (A-F) the top recording is the response to ACh in normal solution. A. Brief, local application of scopolamine completely blocks all actions of ACh (bottom trace, post-scopolamine). B. Application of the muscarinic agonist acetyl- $\beta$ -methacholine causes the typical biphasic response. C and D. Application of the nicotinic agonist DMPP and the cholinergic agonist oxotremorine both cause the hyperpolarizing response only. The fact that DMPP caused the hyperpolarization indicates that although the receptors mediating the excitation of interneurons by ACh are largely muscarinic in character, they may also be somewhat sensitive to traditional nicotinic agonists. E. Applications of the cholinergic agonist dicholine suberate caused relatively weak inhibition, followed by strong slow excitation. F. Pilocarpine did not cause the inhibitory response, but was capable of generating the slow excitatory response. Volume of application of pilocarpine in this cell was four times that of ACh.

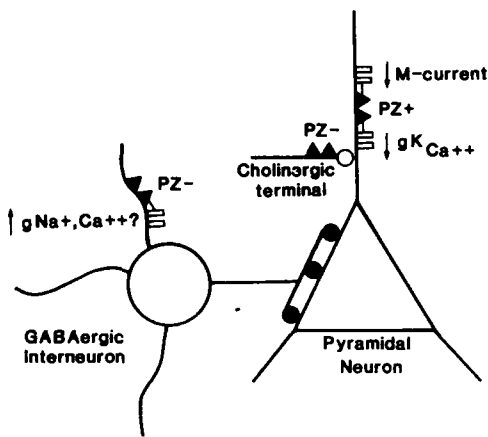


Fig. 5. Schematic diagram of the proposed location and ionic conductances coupled (directly or indirectly) to muscarinic receptors in the cerebral cortex. Cholinergic terminals are found on the dendrites of pyramidal neurones and when activated decrease the M-current and calcium activated potassium conductances ( $g_{KCa^{++}}$ ). This response is selectively sensitive to blockade by pirenzepine (PZ). GABAergic interneurons, on the other hand, respond to ACh with an increase in membrane conductance to at least one or more depolarizing cations (i.e.  $Na^+$ ,  $Ca^{2+}$ ) which causes an excitation. This action of ACh is readily blocked by atropine or scopolamine, but is less sensitive to PZ. Presynaptic receptors regulating the release of ACh have been found by other investigators and are also relatively less sensitive to PZ.

the A PZ sites.<sup>19</sup> Another terminology subclassifies muscarinic sites as  $M_1$  and  $M_2$ , with  $M_1$  showing higher affinity for PZ. The ability of low doses of PZ to selectively block the slow depolarizing response of pyramidal neurones indicates that the latter may be mediated by the  $M_1$  receptor, associated with the A subclass of PZ receptors. The effect of ACh on GABAergic interneurons may be mediated by the  $M_2$  receptor site, associated with the B/C subclass of PZ receptors. However such correlations between receptor subclasses defined through receptor binding experiments and those defined physiologically are as yet only tentative. In any event, the ability of certain agonists to selectively excite either GABAergic or pyramidal neurones provides further evidence for distinct muscarinic receptor-effector mechanisms on these two classes of cortical neurone.

Fig. 5 summarizes, in a schematized drawing, the proposed distribution and actions of postsynaptic muscarinic receptors within the cerebral cortex. Muscarinic synapses and receptors of pyramidal neurones are located primarily upon the dendrites,<sup>30,31</sup> are selectively blocked by PZ (Ref. 24), and suppress the M-current and  $Ca^{2+}$ -activated  $K^+$ -current when activated.<sup>10,15,23</sup> The suppression of these currents results in the enhancement of the pyramidal neurone's response to depolarizing inputs with little or no change in the resting potential or the response to inhibitory inputs. Thus, this action of ACh may function as a 'signal-to-noise' enhancer.<sup>6,21</sup>

In contrast, at least some types of GABAergic interneurons appear to be excited by ACh, i.e. a: a: membrane potentials owing to an increase in membrane conductance for one or more cations. This response is mediated by muscarinic receptor effector complexes which are pharmacologically distinct from those causing slow excitation of pyramidal neurones and are less sensitive to blockade by PZ. Interestingly, a recent investigation has shown that some subclasses (H, or high agonist affinity) of muscarinic receptors may be coupled to  $Na^+$  channels.<sup>32</sup> Such an arrangement might provide a substrate for the rapid muscarinic excitation of interneurons reported here. The response of cortical interneurons to ACh is in some respects similar to that of smooth muscle cells where ACh causes a muscarinic excitation by increasing membrane conductance to one or more cation.<sup>33</sup>

In addition to the above postsynaptic effects, other investigators have found that muscarinic receptors can also be located on presynaptic terminals and, when activated, suppress neurotransmitter release.<sup>34,35</sup> At least some of these receptors (e.g. autoreceptors) are not readily blocked by PZ (Ref. 34).

#### Actions of ACh in the thalamic reticular nucleus

Autoradiographic and pharmacological studies have shown that the subclasses of muscarinic receptors are distributed differentially within the mammalian CNS, with high affinity PZ receptors being more common in forebrain regions (e.g. cerebral cortex, hippocampus, basal ganglia) and low affinity PZ receptors being more common in brainstem/diencephalic regions (e.g. some parts of the thalamus, superior colliculus, motor nuclei).<sup>36,37</sup> With this in mind, we have begun to investigate the actions of ACh on neurones in different brain regions known to contain a particular subclass of muscarinic receptors. One such region is the reticular nucleus (nRt) of the thalamus. The nRt is a collection of

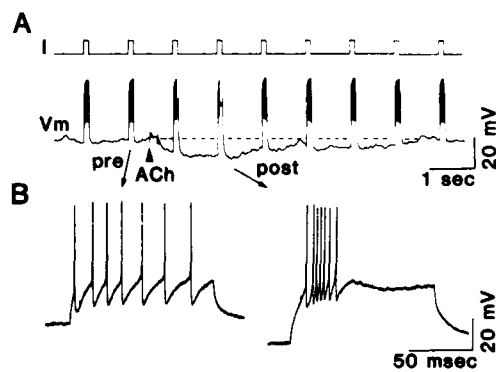


Fig. 6. Effects of application of ACh to neurones within the reticular nucleus of the thalamus (nRt). A. Application of ACh caused a hyperpolarization which shifted the response of the neurone to a depolarizing current pulse from trains of single spikes (B left) to a burst of spikes (B right). Mimicking the effect of ACh on the membrane potential by passing hyperpolarizing current caused a similar change in firing pattern (not shown).

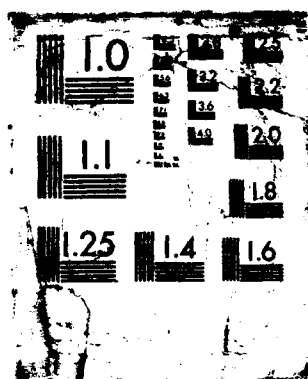
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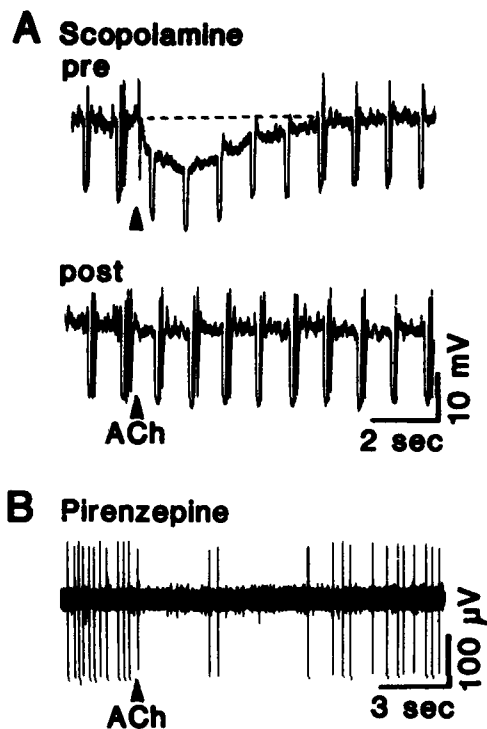


Fig. 7. Pharmacological characteristics of the ACh induced hyperpolarization of nRt neurones. A. Local application of a small amount of scopolamine ( $10 \mu\text{M}$ ) to the surface of the slice completely blocked the response of this neurone to ACh, indicating that it is mediated by muscarinic receptors. B. In contrast, neurones recorded extracellularly in  $10$  or  $100 \mu\text{M}$  pirenzepine (which is more than sufficient to block the slow excitatory responses of cortical pyramidal neurones) failed to block the inhibitory responses of nRt neurones to ACh.

GABAergic neurones which surround the thalamus and receive inputs from virtually all cortico-thalamic and thalamo-cortical axons. This nucleus, which appears to play an important role in the control of thalamic and cortical neuronal activities,<sup>38</sup> receives a cholinergic input from neurones in the brainstem.<sup>39,40</sup> Application of ACh to some thalamic (especially nRt) neurones produces an unusual action: single spike activity is inhibited, but at the same time burst firing and an increase in response to excitatory inputs results.<sup>8,9</sup> Thalamic neurones are known to change their discharge characteristics from single spikes to bursts of spikes when they are hyperpolarized.<sup>41,42</sup> From these observations it seemed likely that ACh would cause a membrane hyperpolarization in nRt neurones. When this hypothesis was tested directly, we found that ACh applications resulted in a membrane hyperpolarization of  $5$ – $20$  mV, and a consequent shift in discharge pattern of nRt neurones from single spikes to

burst firing (Fig. 6). Hyperpolarizing intracellular current injections which mimicked the effects of ACh upon the membrane potential caused a similar shift in firing mode (not shown). This action of ACh was directly on the affected neurones since it was not eliminated when synaptic transmission was blocked with  $\text{Mn}^{2+}$ , low  $\text{Ca}^{2+}$  or TTX.

Further investigations revealed that the ACh-induced hyperpolarization of nRt neurones is associated with an apparent increase in membrane conductance of  $2$ – $4$  nS, which results in a relatively small outward current of approximately  $25$ – $75$  pA. This increase in membrane conductance appears to be specific for  $\text{K}^{+}$  ions since we found that the ACh-induced hyperpolarization has a reversal potential which varies from  $-75$  to  $-103$  mV with changes in extracellular  $\text{K}^{+}$  concentration from  $7.5$  to  $2.5$  mM, as predicted by the Nernst equation. Shifting the equilibrium potential for  $\text{Cl}^{-}$  by impaling the neurones with KCl-filled microelectrodes and iontophoresing  $\text{Cl}^{-}$  intracellularly had no effect upon the ACh-induced response at a time when GABA responses were significantly altered.

The combination of the ACh-induced hyperpolarization and the intrinsic properties of nRt neurones interact to produce unique effects. Like other thalamic neurones,<sup>41,42</sup> those in nRt possess two modes of impulse generation, characterized by single spikes and bursts of  $3$ – $8$  spikes. At normal resting membrane potential or more depolarized levels, the single spike mode is active, while hyperpolarization by  $10$ – $20$  mV deactivates the burst firing mode, allowing subsequent excitatory inputs to activate a fast  $\text{Ca}^{2+}$ -mediated potential which generates a burst of spikes.<sup>41,42</sup> Application of ACh hyperpolarized the neurone towards the equilibrium potential for  $\text{K}^{+}$ , thereby inhibiting single spikes and deactivating the burst firing mechanism. This effect of ACh on the membrane potential subsequently changed the response to excitatory inputs from one or two single spikes to a burst of  $3$ – $8$  spikes. Recorded extracellularly, this effect of ACh would appear as an inhibition of spontaneous single spike activity, but an enhancement of the response to excitatory inputs.<sup>8,9</sup>

#### Cholinergic pharmacology of thalamic reticularis neurones

Local application of the specific muscarinic antagonist scopolamine to nRt neurones completely blocked their responses to ACh (Fig. 7A), while applications of the muscarinic agonist acetyl- $\beta$ -methacholine mimicked the effect of ACh. These results indicate that the actions of ACh on nRt neurones are mediated through muscarinic receptors, as has been reported previously.<sup>8</sup> Bathing the slices in PZ ( $10$  or  $100 \mu\text{M}$ ) failed to block the ACh-induced inhibition (Fig. 7B), and applications of pilocarpine failed to produce it. These results indicate that the ACh-induced hyperpolarization of nRt neurones is mediated through muscarinic receptor-effector complexes which are pharmacologically distinct from those of cortical pyramidal neurones.

In some neurones of the peripheral ganglia ACh causes a slow inhibition and/or slow excitation. The slow excitatory response to ACh is very similar to that of cortical pyramidal neurones since it is due to a decrease in a voltage-dependent conductance<sup>10</sup> and is selectively

antagonized by PZ (Ref. 43). The slow inhibitory response to ACh, on the other hand, closely resembles the ACh-induced hyperpolarization in nRt neurones in that it is caused by an increase in membrane conductance to  $K^+$  (Ref. 43) and is less sensitive to blockade by PZ (Ref. 43).

Our data in conjunction with those from the peripheral nervous system indicate that ACh can affect a number of ionic conductances and that at least some of the receptor-effector complexes mediating these actions can be distinguished pharmacologically. It is not yet known, however, whether the pharmacological differences reported here are due to inherent differences in receptor structure, or to allosteric effects brought about by the coupling of identical receptors to different effector mechanisms. In any event, the available pharmacological data lead to the exciting suggestion that it may be possible to develop specific drugs which will not only selectively activate or inactivate muscarinic receptors located within specific regions of the brain, but which will also distinguish between subtypes of neurones (e.g. pyramidal v. GABAergic) located within these brain regions. Such pharmacological manipulations might be very useful in the treatment of neurological disorders in which abnormalities of cholinergic or GABAergic synaptic transmission might be present such as Alzheimer's disease<sup>44</sup> and epilepsy, respectively.

#### Acknowledgements

We thank Dr Barry Connors for comments on the manuscript, J. Kadis for technical assistance, and C. Joo for help in manuscript preparation. This work was supported by NIH grants NS 06477, 12151 (DAP) and 07331 (DAM). Additional support came from the Pimley Research Fund, the Huntington's Disease Foundation of America, and the Giannini Foundation.

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## 1 Two subtypes of muscarine receptors from rabbit brain in solution *D. D. Flynn and L. T. Potter*

Department of Pharmacology, University of Miami School of Medicine, Miami, FL 33101, USA.

Muscarine receptor 'subtypes' in membranes have been defined on the basis of different agonist and antagonist affinities, sensitivity to guanine nucleotides and *N*-ethylmaleimide (NEM), distinct functions, mechanisms and locations. However distinct receptor proteins have not been distinguished biochemically by size, isoelectric point or reaction with antibodies. We have studied the pharmacological properties of muscarine receptors in digitonin solution in order to determine whether their heterogeneity results from constraints placed by the membrane environment or dissociable effector molecules. Rabbit hippocampus and brainstem, which are rich (~90%) in the putative receptor subtypes  $M_1$  and  $M_2$ , respectively, were used as the source. Muscarine receptors were solubilized with 1% digitonin from membranes prepared from fresh rabbit hippocampus and brainstem in either 20 mM Tris-HCl pH 7.4 buffer containing 1 mM  $MnCl_2$ , or in 50 mM  $Na^+$  phosphate pH 7.4 buffer containing 1 mM  $Na_3EDTA$  and 0.1 mM NEM, by stirring at room temperature for 30 min. Approximately half of the receptors in membranes were obtained in soluble form. Tris- $Mn^{2+}$  yielded soluble receptor preparations showing two interconvertible agonist affinity states in each tissue, whereas  $PO_4^{3-}$ -EDTA stabilized receptors in their lowest agonist affinity states. High affinity carbachol binding to soluble receptors from the brainstem was sensitive to guanine nucleotides and to NEM, while soluble hippocampal receptors remained insensitive to NEM, as in membranes. Soluble receptors in  $PO_4^{3-}$ -EDTA showed markedly decreased affinities for carbachol, consistent with dissociation of effector proteins. In contrast the ratios of apparent  $K_D$ s for the antagonists quinuclidinyl benzilate and pirenzepine were enhanced going from membranes into solution, by factors of 1.5 and 1.8 respectively. These results demonstrate the feasibility of solubilizing high and low affinity forms of muscarine receptor subtypes under appropriate conditions. They also suggest that the removal of effector proteins and some membrane constraints do not yield a homogeneous population of receptor protein.

## 2 Differential inactivation of muscarinic receptor-G protein interactions in rat brainstems by physical and enzymatic means *R. S. Aronstam, B. L. Anthony and G. O. Carrier*

Medical College of Georgia, Augusta, GA 30912, USA.

The influence of guanine nucleotides on muscarinic receptor binding is mediated by a regulatory protein (G protein) which plays an important role in receptor control of intracellular processes. Agonist binding is thought to promote the binding of GTP to the G protein and the subsequent dissociation of an active subunit. Agonists

possess a higher affinity for receptors coupled to G proteins (i.e., in the absence of guanine nucleotides) compared to uncoupled receptors (in the presence of guanine nucleotides).

We have investigated ways of inactivating receptor-G protein interactions in rat brainstem using enzymes, chemical modifying reagents and physical treatments. Receptor-G protein coupling was detected by the ability of 5'-guanylyl-imido-diphosphate (Gpp(NH)p) to decrease carbamylcholine binding affinity as measured in competition experiments with [ $^3H$ ]methylscopolamine.

Essentially two modes of inactivation were identified: (1) elimination of the nucleotide effect with concomitant decrease in agonist binding affinity; and (2) elimination of the nucleotide effect without a concomitant decrease in agonist binding affinity. In the first case, the G subunit is uncoupled from the receptor and/or its normal re-association is blocked. In the second case, the G protein may be inactivated without dissociation from the binding subunit.

Among the agents precipitating the first situation are incubation in media of low pH and heat (50 C for 5 min). Among the agents leading to the second situation are 2° halothane and an endogenous proteolytic activity that is sensitive to serine proteinase inhibitors. Antagonist binding is not permanently affected by any of these treatments. These influences on receptor-G protein interactions and agonist affinity are readily reversible in the case of halothane, but not after exposure to media of low pH or to proteolytic enzymes. The elimination of guanine nucleotide sensitivity by halothane may be due to an alteration of the physical state of the membrane rather than a direct action on binding or regulatory subunits of the receptor complex.

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## 3 Temperature dependence of specific pirenzepine binding to $M_1$ and $M_2$ receptors *N. Mayer*

Biochemistry Department, Dr. K. Thomae GmbH, D-7950 Biberach/Riss, FRG.

The selective muscarinic receptor antagonist pirenzepine binds preferentially to  $M_1$  receptors.<sup>1</sup> The question remained open if the binding selectivity reflects the existence of two different receptors or the presence of two conformational states of one receptor. To get more information on this we tested the temperature sensitivity of the displacement of specific [ $^3H$ ]N-methylscopolamine binding by pirenzepine. The  $IC_{50}$  values were determined in experiments performed with homogenates originating from forebrain and cerebellum.

	cortex $IC_{50}$ (nM)		cerebellum $IC_{50}$ (nM)
	$M_1$	$M_2$	$M_2$
0 C	287	287	811
40 C	91	1004	662

In forebrain where  $M_1$  as well as  $M_2$  receptors are present, the affinity of pirenzepine binding to  $M_1$  receptors was

increased up to 20°C and remained constant up to 40°C. The affinity to  $M_2$  receptors was decreased by increasing the temperature up to 40°C in a linear manner. In the cerebellum, where only low affinity binding sites for pirenzepine were found, the binding affinity was lowered linearly by increasing the temperature. In conclusion, the selectivity of pirenzepine binding is temperature dependent, indicating two molecularly different sites. The low affinity site in cerebellum behaves totally different to the  $M_2$  receptor in forebrain. This indicates the existence of a further muscarinic subtype.

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#### 4 Differences in temperature dependence of muscarinic agonist and antagonist binding to $M_1$ and $M_2$ receptors in different tissues H. A. Ensinger

Boehringer Ingelheim KG, Department of Biochemistry, D-6507 Ingelheim/Rhein, FRG.

According to the concept of  $M_1$  and  $M_2$  muscarinic cholinergic receptor subtypes we studied the receptor binding properties of muscarinic agonists and antagonists in different tissues. The inhibition of [ $^3$ H]N-methylscopolamine binding, (83 Ci mmol<sup>-1</sup>, NEN) in cerebral cortex and heart of the rat (Chbb: THOM, m, 200 g) was investigated at 0°C and at 30°C, respectively. The displacement of [ $^3$ H]Pirenzepine (82 Ci mmol<sup>-1</sup>) from  $M_1$ -receptors in rat hippocampus was investigated at both temperatures, too. The curves were evaluated by a computer fit, based on the law of mass action, delivering  $IC_{50}$  and  $K_D$  values for the unlabelled substances.

The binding in tissues bearing mainly one muscarinic receptor subtype such as  $M_1$  in hippocampus or  $M_2$  in heart showed only slight influence of the changed temperature which is expressed by a  $K_D$  30°C/ $K_D$  0°C ratio between 0.5 and 2.0 for both agonists and antagonists.

In contrast the binding affinity of agonists was clearly affected in rat cerebral cortex (mixed  $M_1$  and  $M_2$  populations). The  $IC_{50}$ -values were shifted to higher concentrations at 30°C. The temperature ratios for agonists resulted in factors between 4-10. Antagonists showed no change, indicated by a ratio of about two. A similar result was obtained with McN-A-343 (4-[m-chlorophenylcarbamoyloxy]-2-butenyl-trimethylammonium chloride), a putative  $M_1$ -receptor agonist.

The temperature dependence of muscarinic agonist and antagonist binding to the muscarinic receptors in rat cerebral cortex is comparable to the sensitivity of their binding behaviour to guanine nucleotides in experiments with  $M_2$ -receptors of the rat heart.

We conclude first, that temperature changes seem only to influence the muscarinic receptors in cerebral cortex, and not the muscarinic receptors in hippocampus and heart of the rat. Second, this method might be used for the characterization of agonists and antagonists.

#### 5 Use of methyl-quinuclidinyl benzilate for the assay of muscarinic cholinergic binding sites M. G. Filbert, C. A. Broomfield, I. J. Dembure, and S. I. Baskin

Divisions of Physiology and Pharmacology, US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010-5425, USA.

A commercially available quaternary derivative of quinuclidinyl benzilate (QNB), 3-hydroxy-1-methyl-quinuclidinium bromide benzilate (Hoffman-La Roche RO-3773, Quarsan, Clidinium Bromide, methyl-QNB) was used as a competing ligand for the determination of specific [ $^3$ H]QNB receptor binding.

Competition between methyl-QNB and [ $^3$ H]QNB for binding sites was examined by incubation of rat forebrain homogenates in 0.05 M phosphate buffer, pH 7.4, for 35 min at 37°C with 0.25 nM [ $^3$ H]QNB and varying concentrations of methyl-QNB. The data obtained were analyzed using a least-squares fit to a logistic function according to the procedure of Waud and Parker<sup>1</sup>. The concentration of methyl-QNB producing 50% inhibition of [ $^3$ H]QNB binding ( $IC_{50}$ ) was determined to be  $3 \times 10^{-9}$  M. The  $IC_{50}$  obtained with unlabelled QNB was  $2.8 \times 10^{-10}$  M, while the  $IC_{50}$ s for scopolamine and atropine were  $7 \times 10^{-6}$  M and  $10^{-8}$  M, respectively<sup>2</sup>. When specific binding of [ $^3$ H]QNB to rat brain homogenates was measured as a function of [ $^3$ H]QNB concentration by using micromolar concentration of either unlabeled QNB or methyl-QNB as the displacing ligand, comparable values for  $K_D$  and  $B_{max}$  were obtained.

Methyl-QNB is a quaternary amine and as such is not subject to the variation of charge resulting from changes in pH. Furthermore, while methyl-QNB displaces all of specifically bound [ $^3$ H]QNB, it is less likely to displace [ $^3$ H]QNB dissolved in the membrane lipid bilayer. Methyl-QNB is not subject to abuse since it has no psychogenic activity and is, therefore, more easily procured than QNB, a controlled substance. Our data suggest that methyl-QNB is a useful ligand for the study of muscarinic receptors.

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#### 6 Apparent affinities of muscarinic antagonists and agonists at binding sites in rat heart and brain S. B. Freedman, M. S. Beer, and E. A. Harley

Meck Sherr and Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Harlow, Essex, CM20 2QR, UK.

It has been suggested that  $M_1$  and  $M_2$  muscarinic receptor subtypes can be distinguished by their affinity for pirenzepine.<sup>1</sup> If such heterogeneity exists it is likely that each subtype will also exhibit multiple affinity states for

muscarinic agonists. We have compared the binding properties of muscarinic receptor binding sites in the rat cerebral cortex ( $M_1$ ) with those in the rat heart ( $M_2$ ).

Binding studies were performed in 20mM Hepes Krebs buffer pH 7.4 using [ $^3$ H]pirenzepine to label heart and brain receptors respectively.

The muscarinic antagonists *N*-methyl scopolamine and atropine displayed similar (nanomolar) affinity in both regions ( $N_H = 1.0$ ). In contrast pirenzepine showed 50-fold selectivity for the brain ( $K_i$ : 746nM heart, 14.6nM brain). A number of other selective muscarinic antagonists have been identified, including dicyclomine ( $K_i$ : 167nM heart, 5.8nM brain) and trihexyphenidyl HCl ( $K_i$ : 93nM heart, 2.2nM brain). Muscarinic agonists displayed lower (micromolar) potencies and flatter displacement curves ( $N_H < 1.0$ ). Quaternary agonists, such as acetylcholine and carbachol, displayed very flat curves ( $N_H \approx 0.5$ ) and showed an apparent selectivity for the heart possibly due to the very high proportion of high affinity sites in this tissue. In contrast, other agonists such as pilocarpine and McN-A-343 (4-[*m*-chlorophenylcarbamoyloxy]-2-butynyl-trimethylammonium chloride) showed steeper curves ( $N_H \approx 0.8$ ) but did not show this apparent tissue selectivity.

Thus muscarinic agents can distinguish between regions on the basis of receptor selectivity and also by recognition of high and low agonist affinity states.

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### 7 Allosteric regulation of muscarinic receptors from rat brain S. B. Freedman, E. A. Harley and L. L. Iversen

Merck Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Harlow, Essex CM20 2QR, England.

It has been suggested that gallamine interacts with muscarinic receptors by an allosteric mechanism.<sup>1</sup> The gallamine site can modify the binding of agonists and antagonists and thus could represent a novel site of drug action. We have utilised the ability of gallamine to modify the dissociation rate of [ $^3$ H]*N*-methyl scopolamine (NMS) binding from rat cerebral cortex membranes to examine a range of drugs for activity at this site.

In the absence of gallamine the dissociation of [ $^3$ H]NMS was complex, with a rapid phase ( $t_{1/2} = 5$  min) and a slow phase ( $t_{1/2} = 15$  min). Gallamine (1 mM) reduced both of these components by 87%. Using a 30 min dissociation in 20mM Hepes Krebs buffer pH 7.4, a number of compounds of diverse biological activity were able to mimic the effects of gallamine (100%). At a concentration of 1mM, these included promethazine (77%), imipramine (77%), verapamil (74%), clozapine (55%), gallopamil (51%), flurazepam (47%), iprindol (46%) and diltiazem (41%). A series of tetraethylammonium derivatives was also shown to exhibit weak activity. The  $EC_{50}$  values of compounds at this site were gallamine = 323 $\mu$ M; pancuronium = 422 $\mu$ M; vecuronium = 562 $\mu$ M; imipramine = 668 $\mu$ M and flurazepam = 1,076 $\mu$ M.

In the presence of buffer of low ionic strength (20mM sodium Hepes pH 7.4) the affinities of these compounds were increased 4–40 fold.  $EC_{50}$ : Gallamine = 11 $\mu$ M; pancuronium = 15 $\mu$ M; vecuronium = 13 $\mu$ M and flurazepam = 237 $\mu$ M.

Thus biological activity at this site showed only limited structural specificity though there was a marked dependence on ionic strength.

#### References

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### 8 Binding profile in rat brain of compound AF-DX 116, a novel cardioselective muscarinic receptor antagonist of the competitive type E. Giraldo, R. Hammer and H. Ladinsky

Department of Biochemistry, Istituto De Angeli S.p.A., 20139 Milan, Italy

The heterogeneity found both between and within some tissues in the binding of pirenzepine, an atypical muscarinic receptor antagonist, has contributed to the classification of muscarinic receptor in  $M_1$  (high affinity for pirenzepine) and  $M_2$  (low affinity for pirenzepine) subtypes. Compound AF-DX 116 [11-(2-[(diethylamino)methyl]-1-piperidinyl) acetyl)-5,11-dihydro-6H-pyrido (2,3-*b*) (1,4) benzodiazepin-6-on) is a novel, competitive muscarinic receptor antagonist capable of discriminating between  $M_1$ -muscarinic receptor subtypes in peripheral tissues, showing high affinity for the  $M_2$ -muscarinic receptor of the myocardium ( $K_D = 100$ nM) and low affinity for the  $M_2$ -muscarinic receptor of the exocrine glands ( $K_D = 4000$ nM). For the  $M_1$ -muscarinic receptor subtype, it shows an intermediate affinity ( $K_D = 700$ nM).

A similar pattern is seen in the brain.

	$M_1$		$M_2$ (cardiac type)		$M_2$ (exocrine gland type)	
	%	$K_D$ (nM)	%	$K_D$ (nM)	%	$K_D$ (nM)
hypothalamus	7	390	41	83	52	3090
medulla-pons	-	-	78	79	22	2900
cerebellum	-	-	89	72	11	2400

In competition experiments v. 0.3 nM [ $^3$ H]*N*-methylscopolamine, AF-DX 116 shows a heterogeneous profile in hypothalamus, medulla-pons and cerebellum. Non-linear least squares regression analysis of the occupancy concentration curves of AF-DX 116 to muscarinic receptor of the three brain regions showed that the data fit best to a three site model in hypothalamus and to a two site model in the medulla-pons and cerebellum. The presence of two major muscarinic receptor subtypes was found in all three regions with affinity values of AF-DX 116 similar to those for the cardiac and glandular receptors.

## 9 The binding of soman antidotes to muscarinic acetylcholine receptors C. A. Broomfield, I. J. Dembure and J. Cuculis

Basic Pharmacology Branch, Pharmacology Division, US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010-5425, USA.

It has been reported that several bis-quaternary compounds not necessarily having an oxime function can be used to treat soman poisoning in mice.<sup>1</sup> The mechanism for this protection is not clear, but the blocking of muscarinic acetylcholine receptors has been proposed.<sup>2</sup> Therefore the muscarinic binding affinity of several compounds was tested in rat brain synaptosomes. Among the compounds tested for muscarinic binding affinity was a series of bis-pyridinium compounds with structures based on that of (1,1-oxodimethylene bis-(4-*tert*-butylpyridinium chloride, SAD-128). These compounds differ from one another only by the number of carbon atoms (from 2–10) separating the two pyridine rings. The concentration of test compound required for 50% displacement of quinuclidinyl benzilate ( $IC_{50}$ ) varied from 6.7 to 280  $\mu$ M, with the binding strength generally increasing with spacer length. The binding affinities of all these compounds were low compared to atropine, and there was not a good correlation between their binding affinity and their reported efficacy against soman.

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## 10 New analogues of pirenzepine: structure-activity relationships of $M_1$ -selective antimuscarinics W. Eberlein, G. Trummlitz, W. Engel and G. B. Schiari\*

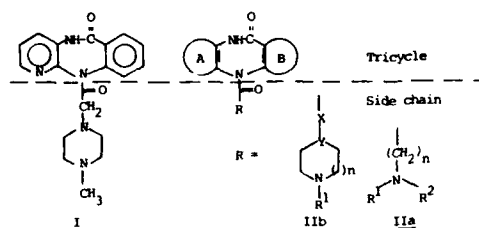
Dr. Karl Thomae GmbH, Birkendorfer Straße 65, D-7950 Biberach 1, FRG.

\* Istituto de Angeli S.p.A. Via Serio 15, 20139 Milan, Italy

Pirenzepine (I) is a unique drug with respect to its biological and pharmacological properties. It is the first selective muscarinic receptor antagonist which has been recently introduced in ulcer therapy and provides safe and unproblematic treatment of gastritis, duodenal and peptic ulcer. The mode of action of pirenzepine is based on a selective blockade of  $M_1$ -receptors leading to selective inhibition of gastric acid secretion in the stomach.

In the course of studies on analogues of pirenzepine we have synthesized a set of compounds (II) and examined these drugs for their selectivity towards  $M_1$  receptors.

The antimuscarinic effects were studied by receptor-binding experiments for muscarinic receptors in cortex and smooth muscle (fundus) of the rat. Selectivity was assessed in terms of the ratio of the binding affinities for two different tissues. Based on these binding experiments the following qualitative structure-activity relationships could be established: receptor affinity is controlled by



both the tricycle and the side chain. Receptor selectivity is controlled similarly by the nature of the tricycle and the side chain but with a dominant influence of the side chain i.e. highly flexible side chains with high degree of freedom about single bonds do not exhibit  $M_1$ -selectivity (IIa). In contrast, side chains with restricted conformational flexibility exhibit selectivity (IIb). Details of structure-activity relationships in regard to activity and selectivity will be presented.

## 11 The effector systems for muscarinic receptors in N1E-115 neuroblastoma cells Michael McKinney and Elliott Richelson

Department of Pharmacology, Mayo Foundation, Rochester, MN 55905, USA.

There are two muscarinic agonist-receptor conformations in N1E-115 cells; each separately mediates a cyclic nucleotide response. Lipooxygenase inhibitors and perturbants of the cellular oxidation-reduction state blocked cGMP formation in these cells. Lipooxygenase inhibitors had no effect on the muscarinic inhibition of prostaglandin-induced cAMP increases. Heat-treated melittin and polycations (e.g., poly-lysine), which stimulated marked increases in cGMP levels with intact cells without lysing them, did not however release substantial amounts of arachidonate (AA) from N1E-115 phospholipids, though having other effects on lipid metabolism. With polycations there was a lag phase before cGMP increased; neuraminidase treatment or increasing the cell number increased this lag. Exogenous AA was inhibitory to receptor-mediated cGMP formation ( $IC_{50} = 45 \mu$ M) and treatment of the cells with exogenous rattlesnake  $PLA_2$ , which increased free AA levels, did not lead to increased cGMP. The inhibitory potency of AA was increased 10-fold by oxidation and certain metabolites of AA, notably 15-hydroxyeicosatetraenoic acid (15-HETE;  $IC_{50} = 8 \mu$ M), were effective inhibitors of cGMP formation. 15-HETE elevated free [ $^3$ H]AA levels and prevented [ $^3$ H]AA esterification into phosphatidylinositol ( $IC_{50} = 7 \mu$ M). [ $^3$ H]15-HETE was rapidly esterified into the inositol phospholipids. The enzyme catalase mediated a rapid and potent ( $IC_{50} = 5 \mu$ M) inhibition of the receptor-mediated cGMP response, without blocking the cAMP response. Catalase also blocked the cGMP response to melittin and polycations, but with lesser potency. Treatment of the cells with pertussis toxin partially blocked the cGMP response and had little effect on the muscarinic cAMP

response. The data suggest that the muscarinic effector system for cGMP: (1) is on or connected with the cell surface; (2) can be activated by aggregation of surface glycoproteins; (3) is sensitive to the redistribution of esterified AA; and (4) initiates a lipid oxidation process that produces a lipid-diffusible second messenger. By contrast, the muscarinic inhibitory cAMP response does not have this profile and, in addition, may not involve guanine nucleotide binding proteins.

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## 12 A comparison of muscarinic receptor occupancy and adenylate cyclase inhibition in the rabbit myocardium *Frederick J. Ehler*

*Department of Pharmacology, UCLA School of Medicine, Los Angeles, CA 90024, USA.*

The muscarinic receptor binding properties of some highly efficacious agonists, partial agonists and competitive antagonists were compared with their effects on adenylate cyclase activity in membranes of the rabbit myocardium. When measured by competitive inhibition of [<sup>3</sup>H]N-methylscopolamine ([<sup>3</sup>H]NMS) binding, the competition curves of the various agonists were adequately described by the ternary complex model. This model assumes that the receptor (R) can bind reversibly with a guanine nucleotide binding protein (N) in the membrane and that the affinity of the agonist for the receptor-guanine nucleotide binding protein complex (RN) is higher than that of the free receptor (R). There was good agreement between the efficacy of each agonist as measured by inhibition of adenylate cyclase and the estimate of the co-operativity between the binding of the agonist receptor complex and the guanine nucleotide binding protein. GTP (0.1 mM) had no significant effect on the binding of [<sup>3</sup>H]NMS but caused an increase in the concentration of the various agonists required for half maximal receptor occupancy. There was good agreement between the concentration of agonist required for half maximal receptor occupancy in the presence of GTP and the reciprocal of the microscopic affinity constant of the agonist receptor complex (DR) estimated from the agonist competition data obtained in the absence of GTP. Also, there was good correlation between efficacy as measured by inhibition of adenylate cyclase and the negatively co-operative effects of GTP on agonist binding.

## 13 Inhibition of carbachol-stimulated GTPase by selective muscarinic antagonists in rat brain *P. H. Franklin and W. Hoss*

*Center for Brain Research, University of Rochester, School of Medicine and Dentistry, Rochester, NY 14642, USA.*

In brain, as well as other tissues, muscarinic receptors are linked to second messenger systems such as activation of

phosphatidylinositol turnover, inhibition of adenylate cyclase and potentiation of cAMP phosphodiesterase. Binding studies show that muscarinic receptors in brain can be distinguished on the basis of affinity for agonists and certain antagonists. Receptors that have high and low affinities for pirenzepine have been designated M<sub>1</sub> and M<sub>2</sub> respectively. These receptors are non-uniformly distributed throughout the brain. Specific proteins that bind and hydrolyse GTP serve to couple receptors to their effectors; however, the relationship between muscarinic receptor subtypes, GTP-binding proteins, and specific effectors remains obscure.

In the present study, we have examined the stimulation of low K<sub>m</sub> GTPase by muscarinic agonists and its inhibition by selective antagonists. Synaptosomal membranes were prepared from rat forebrain homogenates by density gradient centrifugation and lysis in hypotonic media. Low K<sub>m</sub> GTPase was stimulated in a concentration-dependent manner by oxotremorine (EC<sub>50</sub> = 1.6 × 10<sup>-7</sup> M) and carbachol (EC<sub>50</sub> = 5.8 × 10<sup>-6</sup> M), which reached a maximum value at 10<sup>-3</sup> M. The rank order of potency of the inhibition of maximal carbachol-stimulated GTPase was atropine > pirenzepine > gallamine. The IC<sub>50</sub> values were 2.4 × 10<sup>-6</sup> M, 2.2 × 10<sup>-4</sup> M, and 1.75 × 10<sup>-3</sup> M, respectively. The finding that both pirenzepine and gallamine inhibited carbachol-stimulated low K<sub>m</sub> GTPase raises the possibility that GTP-binding proteins are involved in the coupling of more than one of the muscarinic receptor subtypes to their effectors.

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## 14 Uncoupling of muscarinic receptor-mediated biochemical and functional responses by islet-activating-protein (IAP) in the isolated rat atrium *M. Endoh and T. Iijima*

*Department of Pharmacology, Tohoku University School of Medicine, Sendai 980, Japan.*

IAP (pertussis toxin) has in recent years been shown to abolish the muscarinic receptor-mediated inhibition of adenylate cyclase activation (induced by e.g. epinephrine) through modulation of the inhibitory guanine nucleotide regulatory protein (Ni) in a variety of tissues including myocardium.<sup>1</sup> We examined the influence of IAP on the biochemical, electrophysiological and functional changes produced via muscarinic receptor stimulation in the intact rat atrial muscle. The positive inotropic (PIE) and chronotropic (PCE) effects of isoproterenol on left and right atria, respectively, and the accumulation of cAMP produced by isoproterenol in right atria were not modified by IAP-pre-treatment (0.125–1.0 µg/100 g body weight 12–72 h prior to the experiments). Carbachol decreased prominently the PIE and PCE, and cAMP accumulation induced by isoproterenol. IAP-pre-treatment reduced the inhibitory action of carbachol on the isoproterenol-induced PIE and PCE in a dose- and time-dependent manner. Since IAP attenuated also the lowering of cAMP levels (previously elevated by iso-

proterenol) induced by carbachol, attenuation by IAP of carbachol-induced inhibition of the isoproterenol responses may be ascribed to loss of Ni function through ADP ribosylation of the protein by IAP.<sup>1</sup> In the absence of isoproterenol, carbachol caused the negative inotropic (NIE) and negative chronotropic (NCE) effects, which were not associated with changes in tissue cAMP levels but an elevation of cGMP levels. The NIE and NCE of carbachol were also reduced by IAP-pretreatment in a dose- and time-dependent manner. The changes in membrane potentials induced by carbachol (shortening of action potential duration and hyperpolarization) were likewise markedly reduced by IAP-pretreatment. These findings indicate that IAP-treatment uncouples the muscarinic receptor stimulation to the subcellular processes not only through the dysfunction of Ni (at the level of adenylate cyclase) but also through yet undefined mechanism which is independent of cAMP metabolism.

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### 15 Selective effects on muscarinic responses at the level of receptor-effector coupling *Joan Heller Brown and David Goldstein*

*Division of Pharmacology M-013 H, UC San Diego, La Jolla, CA 92093, USA.*

Cholinergic agonists inhibit cAMP formation and stimulate phosphoinositide (PI) hydrolysis in embryonic chick heart cells. We have used this preparation to compare the receptors coupled to these two responses. We reported earlier<sup>1</sup> that carbachol inhibits cAMP formation with an  $EC_{50}$  of 0.2  $\mu$ M, whereas the  $EC_{50}$  of carbachol for stimulation of PI hydrolysis is 20  $\mu$ M. We have now used receptor inactivation with propylbenzylcholine mustard to remove receptor reserve and determine the true  $K_A$  of the receptor for agonist. The  $K_A$  values thus determined are the same (30-40  $\mu$ M) for both responses, indicating that the same receptor state mediates both responses, and that differences in receptor reserve account for the discrepancy in the  $EC_{50}$  values.

The possibility that  $M_1$ -selective drugs preferentially affect the PI-coupled receptor was tested. Schild analysis shows the  $K_A$  of pirenzepine as an antagonist of the muscarinic effect on PI hydrolysis to be 240 nM and that for cAMP formation to be 60 nM. For atropine both values are 1-2 nM. The agonists McN-A-343 (4-[*m*-chlorophenylcarbamoyloxy]-2-butenyl-trimethyl ammonium chloride) and A11R 602 do not stimulate PI hydrolysis but are partial agonists for lowering cAMP. These data are inconsistent with the idea that an  $M_1$  receptor is selectively coupled to PI hydrolysis.

The muscarinic receptor regulating cAMP formation is coupled to adenylate cyclase through a nucleotide binding protein,  $N_1$ . Pertussis toxin ribosylates this protein and blocks muscarinic effects on cAMP formation in chick heart cells at concentrations between 1 and 100 ng ml<sup>-1</sup>. Muscarinic effects on PI hydrolysis are not inhibited by

pertussis toxin. In contrast, PMA, a phorbol ester, prevents muscarinic stimulation of PI hydrolysis in chick heart cells but does not block muscarinic inhibition of cAMP. Neither phorbol ester nor pertussis toxin directly affect ligand binding to the muscarinic receptor. We propose that there are differences in the receptors, manifest at the level of receptor effector coupling, and that these differences can be used to selectively activate responses with partial agonists, or to block them with pertussis toxin or phorbol ester.

#### Reference

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### 16 Activation of phosphoinositide phosphodiesterase by guanine nucleotides in membranes from rat brain *R. A. Gonzales and F. T. Crews*

*University of Florida Medical School, Gainesville, FL 32610, USA.*

Receptor stimulated inositol phospholipid hydrolysis is a signal transduction mechanism for many hormones and neurotransmitters. Although recent data suggest that agonists stimulate the phosphodiesteratic cleavage of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>), the exact mechanism whereby agonist occupied receptors activate a PIP<sub>2</sub> specific phospholipase C remains unclear. We have investigated the possibility that guanine nucleotide binding proteins may be involved in the signal transduction process from agonist-receptor complex to phospholipase C. Rat cerebral cortical membranes were prepared from slices which had been incubated with [<sup>3</sup>H]inositol or <sup>32</sup>P, to label inositides. Slices were homogenized gently with a glass-glass homogenizer in a Krebs-Ringer buffer containing 1 mM EDTA, and the homogenate was centrifuged at 1000 × g for 20 min. The resulting pellet was then resuspended in 20 mM Tris and washed again with Tris. The release of [<sup>3</sup>H]inositol phosphates or [<sup>32</sup>P]inositol phosphates from the prelabelled membranes was determined by separating the water soluble organic phosphates on an anion exchange column. 5'-guanylylimidodiphosphate (GppNHp) (100  $\mu$ M), a nonhydrolyzable analog of GTP, stimulated the production of [<sup>3</sup>H]inositol phosphates approximately 300% over control levels. The kinetics of the stimulation by GppNHp was rapid with a maximal effect reached within 2 min. Inositol trisphosphate production was enhanced 30-fold compared to smaller increases for inositol bisphosphate and inositol phosphate. Analysis of the [<sup>32</sup>P]inositides also showed that PIP<sub>2</sub> was hydrolyzed in response to GppNHp stimulation. Guanine nucleotides were more effective than adenine nucleotides. In addition, the GppNHp effect was independent of Ca<sup>2+</sup> at concentrations less than 100  $\mu$ M. These results suggest that guanine nucleotides can activate an inositide specific phosphodiesterase. The kinetic characteristics and Ca<sup>2+</sup> independence of the phospholipase C suggest that the enzyme is the same one which is involved in receptor activated inositide breakdown in other tissues. Furthermore, the activation of this enzyme by guanine nucleotides suggests that guanine

nucleotide binding proteins may be involved in the coupling of receptor stimulation to the phosphoinositide phosphodiesterase for this signal transduction mechanism.

Supported by NIAAA AA06069.

### 17 Muscarinic receptor mediated inositol phospholipid metabolism in guinea-pig parotid gland, ileum and cortex B. Ek and S. R. Nahorski\*

Department of Physiology, University of Göteborg, Sweden. \*Department of Pharmacology and Medical Therapeutics, University of Leicester, Leicester, U.K.

There is now substantial evidence that muscarinic receptor stimulation induces a rapid hydrolysis of inositol phospholipids in many tissues. We have examined this response in guinea-pig cerebral cortex, ileum and parotid gland in view of indications of receptor heterogeneity ( $M_1$  and  $M_2$ ) among such tissues. Measurement of [ $^3$ H]inositol phosphate accumulation ([ $^3$ H]IP) was made in tissue slices after pre-incubation with [ $^3$ H]inositol in the presence of  $Li^+$  (5 mM).

Carbachol and oxotremorine stimulated  $^3$ H-IP in all tissues though significant differences were observed in apparent  $EC_{50}$  values for the full agonist carbachol and the maximal response to the partial agonist oxotremorine.

	$EC_{50}$ ( $\mu$ M)		oxotremorine (%) max response	$K_i$ (nM)	
	carbachol	oxotremorine		atropine	pirenzepine
parotid gland	3.7 $\pm$ 0.49	0.65 $\pm$ 0.16	31.8 $\pm$ 2.15	0.45 $\pm$ 0.17	60.0 $\pm$ 16.2
ileum	20.2 $\pm$ 4.32	0.95 $\pm$ 0.36	17.8 $\pm$ 1.50	0.34 $\pm$ 0.06	32.6 $\pm$ 13.9
cerebral cortex	40.1 $\pm$ 7.27	0.92 $\pm$ 0.26	8.6 $\pm$ 2.15	0.74 $\pm$ 0.08	23.2 $\pm$ 15.6

The response to carbachol was potently blocked by atropine with very similar affinity in each tissue. The  $M_1$  antagonist pirenzepine also inhibited the response though with a slightly high affinity in cortex. [ $^3$ H]NMS binding to membranes of these tissues revealed complex displacement by pirenzepine in cortex best described by a two-site interaction ( $K_H$ , 20 nM,  $K_L$ , 380 nM) but by a single low affinity site (575 nM) in ileum and parotid gland. The data suggest that there may be different coupling of muscarinic receptors to phospholipase C and also that they are not simply compatible with the concept that  $M_1$ - but not  $M_2$ -receptors are linked to this enzyme.

### 18 Potent and selective muscarinic ( $M_1$ ) antagonists L. Noronha-Blob, J. Ferkany, D. Costello, V. Lowe, W. Rzeszotarski, W. Kinnier and D. U'Prichard

Nova Pharmaceutical Corp., Baltimore, MD 21224, USA.

Previous studies suggested that the muscarinic ( $M$ ) receptor antagonists, 3-quinuclidinyl atrolactate (QNA), and 3-quinuclidinyl xanthene-9-carboxylate (QNX), were

substantially selective for muscarinic cholinergic receptors ( $M_1$ -AChR) (Rzeszotarski *et al.*; VIIIth Int. Symp. on Med. Chem., 1984). Four isomers of QNA, (RR, RS, SR, SS), and (R)-QNX were tested for potency and selectivity at  $M$ -receptors using receptor binding and receptor-coupled functional assays. (RR)-QNA ( $K_i$  = 0.6 nM) was more potent than (RS) ( $K_i$  = 3.9 nM) to inhibit [ $^3$ H]-pirenzepine binding to bovine striatal membranes ( $M_1$  selective). The (SR) and (SS) isomers were essentially inactive ( $K_i$  = > 1000 nM). (RR)-QNA was also more potent ( $K_i$  = 6.3 nM) than (RS) ( $K_i$  = 40.3 nM), (SR) ( $K_i$  = 776 nM) and (SS) ( $K_i$  = 602 nM) to inhibit [ $^3$ H]QNB binding to rat cardiac membranes ( $M_2$ -selective). In phosphatidyl inositol hydrolysis studies (PI), (RR)-QNA ( $IC_{50}$  = 5.7 nM) was 4-, 11-, and 20-fold more potent than the (RS) ( $IC_{50}$  = 22.5 nM), (SR) (65.0 nM) and (SS) ( $IC_{50}$  = 114.5 nM) isomers to reverse carbachol- ( $10^{-3}$  M) induced stimulation of PI turnover in rat cortical slices ( $M_1$ -mediated).  $K_i$  values for (RR) and (RS)-QNA were 0.9 nM and 5.7 nM, respectively. The same rank order of potency (RR) QNA > (RS) > (SR) > (SS) was obtained for the reversal of oxotremorine- ( $10^{-4}$  M) induced inhibition of adenylate cyclase in rat heart membranes ( $M_2$ -mediated).  $K_i$  values for (RR) and (RS)-QNA were 152.5 nM and 1460 nM, respectively. (SR) and (SS) were > 100-fold less potent than (RR). Taken together, these data show that (RR) and (RS)-QNA exhibit a 10-fold greater selectivity at the ( $M_1$ ) receptor in binding studies and a substantially greater selectivity (150-300 fold) in the  $M_1$ -receptor coupled functional studies. The  $K_i$  values of (R)-QNX to inhibit [ $^3$ H]QNB and [ $^3$ H]pirenzepine binding were 0.29 nM and 0.2 nM, respectively. (R)-QNX was 6-fold more selective in the PI ( $K_i$  = 1.2 nM) than in the AC ( $K_i$  = 6.9 nM) assay systems. (RR) and (RS)-QNA and (R)-QNX may be useful  $M$ -antagonists for muscarinic receptor subtype identification and function.

### 19 Stimulation of a muscarinic receptor increases force of contraction and phosphatidylinositol turnover in the feline heart P. Gjöström, H. Hårding, B. Jacobsson and L. Ransnäs

Department of Pharmacology and Biochemistry, Hässle Cardiovascular Research Laboratories, S-431 83 Mölndal, Sweden and Department of Medicine, University of Göteborg, Sweden.

A positive inotropic effect is sometimes obtained with ACh, i.e. upon cessation of vagal stimulation or in the presence of atropine (see Ref. 1); it has now been further studied with the agonists carbachol and oxotremorine.

In cat papillary muscle, paced at 0.5 Hz at  $L_{max}$ , carbachol increased force of contraction by  $35 \pm 6\%$  above basal active tension with an  $EC_{50}$  value of  $8.3 \pm 0.7 \times 10^{-7}$  M ( $n=6$ ; mean  $\pm$  S.E.). Corresponding values for the reference substance isoprenaline were  $118 \pm 29\%$  and  $1.7 \pm 0.6 \times 10^{-6}$  M. Any increase produced by oxotremorine was usually smaller ( $0 \leq 10\%$ ) obtained at high concentrations,  $10^{-5}$ – $10^{-4}$  M, and proper concentration-effect curves were not obtained. The increase caused by carbachol remained after pretreatment with

phenoxybenzamine  $10^{-6}$ M, propranolol  $3 \times 10^{-8}$ M or hexamethonium  $10^{-3}$ M, while atropine  $3 \times 10^{-8}$ M increased the  $EC_{50}$  value for the carbachol produced inotropic response to  $1.3 \pm 1.2 \times 10^{-4}$ M ( $n=5$ ).

Phosphatidylinositol turnover in cat ventricular tissue was increased by  $47 \pm 5\%$  above basal level at  $10^{-4}$ M of carbachol, but not by oxotremorine, when measured as formation of [ $^3$ H]myo-inositol-1-phosphate. In competition binding experiments with [ $^3$ H]quinuclidinyl benzilate ( $n=8$  for each) carbachol binding amounted to 41% to a high ( $K_D 3 \mu$ M) and 59% to a low affinity site ( $K_D 150 \mu$ M), while for oxotremorine the data were 47% ( $K_D 0.03 \mu$ M) to a high and 53% ( $K_D 1 \mu$ M) to a low affinity site. In the presence of 5'-guanylimidodiphosphate,  $10^{-4}$ M, carbachol still bound to the high affinity site by 70%, while oxotremorine only bound to its low affinity site.

The results indicate a positive inotropic effect of muscarinic receptor stimulation, possibly linked to phosphatidylinositol turnover and may involve a muscarinic receptor insensitive to affinity shifts by guanine nucleotide.

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## 20 Inositol uptake by cultured myenteric neurons N. J. Buckley

National Institute of Mental Health, Laboratory of Cell Biology, Bethesda, MD 20205, U.S.A.

Muscarinic receptors have been localized on the surface of 10-20% of cultured myenteric neurons.<sup>1</sup> Many muscarinic responses are known to be accompanied by an increase in the hydrolysis of inositol phospholipids (PI response). We were thus interested to discover if we could similarly detect a subpopulation of cultured myenteric neurons expressing a PI response. In the present study, autoradiographic procedures have been used to localize sites of [ $^3$ H]inositol incorporation into cultures of myenteric ganglia. Myenteric neurons present in explant cultures conserve many of their chemical, morphological and electrophysiological properties expressed *in situ* and offer an experimental system whereby intact living cells can be labelled and identified with relative ease. Explant cultures of myenteric ganglia were prepared from newborn guinea-pig caeci and were labelled by a modified version of the method of Anderson *et al.*<sup>2</sup> Cultures were incubated according to three regimes: (1)  $1 \mu$ M [ $^3$ H]inositol (20 Ci mmol<sup>-1</sup>); (2)  $1 \mu$ M [ $^3$ H]inositol +  $100 \mu$ M carbachol; (3)  $1 \mu$ M [ $^3$ H]inositol +  $100 \mu$ M atropine. All incubations were carried out in culture growth medium (without serum) for 2 h at 37°C. After labelling, cultures were fixed by cold glutaraldehyde and osmic acid, dipped in nuclear emulsion and exposed for 3-6 weeks. No specific uptake was seen in cultures incubated in [ $^3$ H]inositol only. In contrast, cultures incubated in [ $^3$ H]inositol and carbachol showed specific uptake by many of the myenteric neurons. Autoradiograph grains were distributed over the cell body and proximal regions of the neurites. In the presence of atropine, only background labelling was seen. These results indicate that a sub-

population of cultured myenteric neurons incorporate [ $^3$ H]inositol in response to muscarinic stimulation. Although the relationship between this population of cells and those expressing a muscarinic binding site remains to be directly established, these data are consistent with the notion that a subpopulation of myenteric neurons express muscarinic receptors and that muscarinic stimulation of these neurons is accompanied by a PI response.

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## 21 Muscarinic receptor-activated signal transduction in PC12 cells L. M. Vicentini, F. Di Virgilio, T. Pozzan and J. Meldolesi

Department of Pharmacology, University of Milano, and Institute of General Pathology, University of Padova, Italy.

Muscarinic receptors exist in a rat pheochromocytoma neurosecretory (PC12) cells and their number increases greatly (5-10-fold) after nerve growth factor-induced differentiation. PC12 cells are therefore an ideal system to study the muscarinic receptor-activated signal transduction. Exposure of differentiated PC12 cells to the muscarinic agonist carbachol (together with hexamethonium to block nicotinic receptors) induced within seconds the hydrolysis of phosphoinositides (PI; mainly of the phosphatidylinositol-4,5-bisphosphate) and a rise in cytosolic Ca ( $[Ca^{2+}]_i$ ) as measured by the quin-2 technique. The muscarinic-dependent  $[Ca^{2+}]_i$  rises were due to both redistribution from internal stores and influx from the extracellular medium. Carbachol had identical efficacy and potency for the stimulation of both the inositol phosphates release and  $[Ca^{2+}]_i$  rises ( $IC_{50} \sim 20 \mu$ M) and the two responses were inhibited in a parallel manner by atropine and pirenzepine with a  $K_i$  of 1.5 and 16 nM, respectively. Moreover, the carbachol-induced PI hydrolysis was still observed when cells, incubated in  $Ca^{2+}$ -free medium, were depleted of their  $Ca^{2+}$  stores by pretreatment with ionomycin. Thus, in PC12 cells PI hydrolysis is independent from both extracellular  $Ca^{2+}$  and  $[Ca^{2+}]_i$  rises.

The existence of two types of muscarinic receptors, one coupled to PI breakdown, the other to adenylate cyclase inhibition, has been proposed. In membrane preparations of our PC12 cells a muscarinic receptor-mediated inhibition of adenylate cyclase has not been detected. Moreover, in intact PC12 cells, carbachol treatment did not modify the level of cAMP.

Muscarinic agonist-induced hydrolysis of PI generates on one hand inositoltrisphosphate which mobilizes  $Ca^{2+}$  and on the other hand diacylglycerol (DAG) which activates protein kinase C. We found that pretreatment of the cells with a phorbol ester which is a potent activator of protein kinase C, inhibited  $[Ca^{2+}]_i$  rises and inositol phosphates release stimulated by carbachol, without affecting muscarinic receptor binding. These results indicate that in PC12 cells the muscarinic receptor coupled to PI breakdown is under the feedback control of

the metabolites (DAG) generated as a consequence of its activation.

## 22 Muscarinic receptor mediating $K^+$ conductance increase of parabrachial neurons has a low affinity for pirenzepine T. M. Egan and R. A. North

Massachusetts Institute of Technology, Cambridge, MA 02139, USA.

We made intracellular recordings from neurons of the nucleus parabrachialis contained within 300  $\mu\text{m}$  slices of rat pons *in vitro*. Acetylcholine hyperpolarized the membrane when it was added to the superfusate; effective concentrations were 100 nM–30  $\mu\text{M}$  in the presence of neostigmine (1  $\mu\text{M}$ ), and 10–100  $\mu\text{M}$  in the absence of neostigmine. Muscarine also hyperpolarized parabrachial neurons, the effect being observed in 21 of 32 cells. The muscarinic hyperpolarization persisted during superfusion of low  $\text{Ca}^{2+}$ , high  $\text{Mg}^{2+}$  solutions, implying that the effect of acetylcholine did not result from the liberation of another neurotransmitter.

The hyperpolarization was completely blocked by atropine and scopolamine (3–100 nM), but not by nicotine (50  $\mu\text{M}$ ) or hexamethonium (400  $\mu\text{M}$ ). Pirenzepine did not affect the muscarinic hyperpolarization in concentrations less than 300 nM, but caused a parallel shift to the right of the muscarine dose-response curve at concentrations greater than 300 nM. Schild plots were constructed from the muscarine hyperpolarizations of individual neurons in the presence of several concentrations of pirenzepine. The pirenzepine dissociation equilibrium constant was about 600 nM.

The muscarinic hyperpolarization was accompanied by an increase in membrane conductance. The amplitude of the hyperpolarization was linearly dependent on the membrane potential, having zero amplitude at a potential which changed with the logarithm of the external  $K^+$  concentration. In control solutions ( $[\text{K}_o]=2.5$  mM), the reversal potential was –104 mV. These findings indicate that the muscarinic hyperpolarization resulted from an increase in the conductance of the membrane to  $K^+$ .

In summary, acetylcholine hyperpolarizes parabrachial neurons by opening  $K^+$  channels. The muscarinic receptor involved in this hyperpolarization has a low affinity for pirenzepine, indicating that it belongs to the  $M_2$  category. This contrasts with the muscarinic receptor subtype involved in the closure of membrane  $K^+$  channels, which has a high affinity for pirenzepine.

## 23 Selective agonists and antagonists in the variety of muscarinic responses in the single cells of rabbit sympathetic ganglia H. Kobayashi and S. Mochida

Department of Physiology, Tokyo Medical College, Shinjuku-ku, Tokyo 160, Japan.

Single neurons, enzymatically isolated from rabbit superior cervical ganglia and solitarily grown in the

culture medium for 4–6 days, provide a suitable opportunity to study the nature of muscarinic responses to exogenously administered agonists, independent from any possibility of indirect action they would introduce via complex interneuronal networks within the intact ganglion. Neurons thus prepared were studied by conventional intracellular recording techniques. Four different kinds of electrical changes were shown to be detectable: (1) acceleration of the repolarizing phase of action potential with remarkable shortening of after-spike hyperpolarization; (2) slow depolarization (DP) with increase in membrane conductance ( $G_m$ ) independent of membrane potential ( $V_m$ ); (3) DP with decrease in  $G_m$  at  $V_m$  positive to about –65 mV; and (4) decrease in  $G_m$  (often associated with hyperpolarization, HP) possibly independent of  $V_m$ . Acetylcholine (in the presence of D-tubocurarine), DL-muscarine and McN-A-343 (4-[*m*-chlorophenyl-carbamoyloxy]-2-butyryl-trimethyl ammonium chloride) all produced the above responses in a given cell; responses 1 and 2 were most readily inducible by the low concentrations (e.g., 10 nM for acetylcholine and 1  $\mu\text{M}$  for McN-A-343), followed by responses 3 and 4 with much higher doses of agonists. Arecoline induced only responses 2–4 at lower concentrations than that of acetylcholine. The above four responses were antagonized by QNB (quinuclidinyl benzilate; 1  $\mu\text{M}$ ) and pirenzepine (10  $\mu\text{M}$ ). Gallamine (as high as 50  $\mu\text{M}$ ) did not antagonize the above four responses. Pharmacologically, the observed muscarinic responses all appear to be classified as  $M_1$  type. In view of functional significance, responses 1 and 3 in combination may help the cell to easily maintain higher frequency of repetitive firing. Response 2 may work to initiate the muscarinic DP when the cell is at the resting  $V_m$  (usually at the level of –65 mV or more). Responses 2 and 3 would be major components of physiological slow excitatory postsynaptic potential (s-EPSP). With higher concentrations of acetylcholine, response 4 introduces additional HP component, so limiting the excessive DP. However, whether this constitutes (at least a portion of) the slow inhibitory postsynaptic potential (s-IPSP) is still inconclusive.

## 24 Antagonist-like action of oxotremorine on a muscarinic receptor in guinea-pig olfactory cortex *in vitro* S. H. Williams and A. Constanti

MRC Neuropharmacology Group, Department of Pharmacology, The School of Pharmacy, 29 39 Brunswick Square, London WC1N 1AX, UK.

Oxotremorine appears to act as a partial agonist at the ganglionic muscarinic acetylcholine receptor (mAChR), a putative  $M_1$  type receptor.<sup>1</sup> We have investigated the pharmacology of oxotremorine on a mammalian brain slice preparation *in vitro*. Surface slices of guinea-pig olfactory cortex (OC) were incubated in Krebs' medium at 23–25 °C. Field potentials evoked by electrical stimulation of the lateral olfactory tract were recorded from the pial surface using extracellular electrodes. All drugs were bath-applied. Addition of muscarine, carbachol or acetylcholine (10–200  $\mu\text{M}$ ) produced a dose-dependent depression of the evoked field potential that was blocked by

atropine. When oxotremorine was applied (0.001–100  $\mu\text{M}$ ) little or no muscarinic agonist action was detected. However, carbachol or muscarine doses were much less effective when applied after test oxotremorine doses, an effect which was reversed by extensive washing (2 h). Preincubation with oxotremorine (15 mins) produced rightward shifts of the carbachol dose-response curve, and yielded a Schild plot with a slope of  $0.99 \pm 0.15$  (mean  $\pm$  SEM) and a  $pA_2$  of  $6.3 \pm 0.1$ . A similar preincubation with a threshold (1  $\mu\text{M}$ ) dose of carbachol did not reduce subsequent supra-threshold carbachol doses.

These results suggest that oxotremorine can act in an antagonistic manner at this olfactory cortex muscarinic receptor, which is probably presynaptically located.<sup>2</sup> The antagonism appeared competitive since a Schild plot slope of near unity was obtained. The  $pA_2$  value indicated a relatively high affinity for muscarinic acetylcholine receptor although the lack of agonist action suggests a low efficacy. The possibility of a desensitizing block seems unlikely since the effects of oxotremorine could not be mimicked by a 'full' agonist using the same protocol.

SHW is supported by the MRC.

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## 25 Pharmacological analysis of the inhibition of vagal stimulated acid secretion by pirenzepine and atropine in the isolated mouse stomach J. W. Black and N. P. Shankley

Rayne Institute, King's College School of Medicine and Dentistry, London SE5 9NU

Pagani *et al.* (1984)<sup>1</sup> concluded that the muscarinic receptors involved in the vagal stimulation of acid secretion in the isolated mouse stomach were of the  $M_1$  subtype in contrast to the  $M_2$  subtype present on oxyntic cells. This conclusion was based on the  $EC_{50}$  values estimated for the inhibitory effects of pirenzepine and atropine on single responses to bethanechol and electrical stimulation. We have extended their analysis examining the effects of pirenzepine and atropine on fully-defined frequency-effect curves on an improved isolated, mouse stomach assay.<sup>2</sup> Both atropine and pirenzepine produced a concentration-dependent inhibition of vagal-stimulated gastric acid secretion in a manner consistent with a model describing the competitive antagonism of endogenous acetylcholine, assumed to be released by vagal stimulation. The affinity estimates for pirenzepine and atropine were similar to those previously estimated<sup>1,2</sup> with 5-methylurmethide ( $pK_B = 6.67, 7.78$ , respectively) and McN-A-343 (4-[m-chlorophenyl carbamoyloxy]-2-butyryltrimethyl ammonium chloride) (6.69, 7.90) stimulated gastric acid secretion.

We conclude that the muscarinic receptors involved in vagal stimulation are homogeneous with those on oxyntic cells. The alternative explanation we previously<sup>4</sup> offered for pirenzepine's relatively selective inhibition of acid secretion, compared to atropine, is still tenable: atropine,

due to high lipophilicity, is lost from the receptor compartment through the gastric secretion, causing apparent underestimation of its affinity relative to pirenzepine.

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## 26 Selective inhibition of vagally induced acid secretion by pirenzepine F. Pagani, C. Ciprandi, and A. Giachetti

Department of Pharmacology, Istituto De Angeli, 20139 Milan, Italy

Potent inhibition of the acid secretion elicited through reflex vagal stimulation, coupled with weak antagonism of secretory responses to muscarinic agonists is the characteristic profile of pirenzepine, a selective  $M_1$  antagonist. These observations have lead us to postulate a dual muscarinic mechanism for regulating acid secretion: a neural one, mediated by  $M_1$  subtypes, operant at the enteric neurones level, and second one, involving  $M_2$  subtypes, in the activation of parietal cells. In order to support this concept we have done experiments on the lumen perfused stomach of the anaesthetized rat in which acid secretion was elicited either by stimulation of the vagus (3–6 Hz, 2 ms, 10 V, for 10 s) or by infusion of the agonists, bethanechol or oxotremorine. To ascertain that bethanechol stimulated only parietal cell receptors, in few experiments, neural activity was abolished by i.v. tetrodotoxin. Pirenzepine and atropine were also compared for their ability to inhibit basal acid output in chronic fistula rats, in which secretion is dependent on the vagal tone.

	chronic fistula	ED <sub>50</sub> (nmol kg <sup>-1</sup> i.v.) perfused stomach vagal bethanechol oxotremorine		
pirenzepine	217	419	139	102
atropine	49	62	4.5	3.8
ratio:				
pirenzepine	4	7	31	28
atropine				

As shown by the tabulated data pirenzepine approached the potency of atropine in inhibiting secretion stimulated by the vagus (direct stimulation or chronic fistula rats). In contrast, pirenzepine was approximately 30-time, less potent than atropine in antagonizing direct parietal cell stimulation. These results strengthen the hypothesis of the dual muscarinic control on acid secretion.

## 27 M<sub>1</sub> muscarinic antagonists selectively inhibit vagally mediated acid secretion A. Giachetti, O. Angelici, R. Micheletti and A. Schiavone

Department of Pharmacology, Istituto De Angeli, 20139 Milan, Italy.

Muscarinic receptor subtypes, M<sub>1</sub> and M<sub>2</sub>, mediate the cholinergically-induced acid secretion. M<sub>1</sub>, predominantly localized in the enteric neurones, modulate vagal secretion, whereas M<sub>2</sub>, distributed on parietal cells, activate the final secretory process. Evidence for this mechanism has been obtained by using pirenzepine which potently inhibits vagal induced secretion but weakly antagonizes direct parietal cell stimulation. We have examined dicyclomine, a lipophilic drug possessing M<sub>1</sub> selectivity in binding to membranes, with the aim of determining whether M<sub>1</sub> selectivity would be reflected on a functional parameter. Antisecretory potency was evaluated in the mouse stomach in which acid secretion was induced by bethanechol or by electrical field stimulation. IC<sub>50</sub> were calculated on the inhibition (%) of the peak output.

	mouse stomach	
	IC <sub>50</sub> (μM ± 95% confidence limits)	
	bethanechol	field stimulation
atropine	0.03 (0.02-0.04)	0.07 (0.05-0.09)
pirenzepine	0.84 (0.57-1.22)	0.33 (0.23-0.49)
dicyclomine	11.80 (8.10-17.20)	2.56 (1.93-3.38)

Both pirenzepine and dicyclomine preferentially antagonized secretion evoked through stimulation of the intrinsic neurones. Conversely, atropine was manifestly more potent in inhibiting bethanechol activation of parietal cells. The high lipophilicity of dicyclomine did not affect its ability to discriminate between neurally evoked and parietal cell stimulation of acid secretion, although it may lead to an underestimation of its antisecretory activity. These results support the hypothesis that M<sub>1</sub> muscarinic subtype, sensitive to selective antagonists, is involved in neurally evoked acid secretion.

## 28 Carbachol potentiation and inhibition of histamine-stimulated acid secretion by isolated rat parietal cells G. C. Rosenfeld

The University of Texas Medical School, Houston, TX, 77025, U.S.A.

Histamine, or its second messenger cAMP, and cholinergic agonists act in concert to potentiate isolated rat parietal cell acid secretion, as measured indirectly by the accumulation of [<sup>14</sup>C]aminopyrine (AP). A more detailed analysis showed that in the presence of 10<sup>-4</sup> M histamine, carbachol which had no effect of its own, potentiated [<sup>14</sup>C]aminopyrine accumulation up to a concentration of 3.0 μM. However, at higher concentrations carbachol inhibited the potentiated response in both unenriched (15%) and enriched (60%) parietal cell populations. Maximum inhibition by carbachol was at 100 μM (70% of

maximum potentiated response) and half-maximum inhibition was at approximately 20 μM. Carbachol (100 μM) inhibition of [<sup>14</sup>C]aminopyrine accumulation was less pronounced (20-40%) when 10<sup>-4</sup> M dibutyryl cAMP was substituted for histamine. In the presence of a potentiating concentration of carbachol (1 μM) there was no change in the potency of histamine (EC<sub>50</sub> = 4.0 μM). However, 10 μM-100 μM carbachol decreased the potency of histamine (EC<sub>50</sub> = 50 μM), and also decreased the maximum potentiated response (65%). Inhibition of [<sup>14</sup>C]aminopyrine accumulation by carbachol (100 μM) was prevented by both pirenzepine and atropine at a potency ratio of 200:1, indicative of an effect of carbachol at parietal cell muscarinic M<sub>2</sub>-type receptors shown previously to also mediate carbachol's potentiating action.<sup>1</sup> Reducing the extracellular concentration of Ca<sup>2+</sup> from 2 mM to 3 μM prevented the inhibitory action of carbachol. Under this condition the EC<sub>50</sub> for carbachol potentiation of histamine-stimulated [<sup>14</sup>C]aminopyrine accumulation was 7 μM. The results of this study support a possible cholinergic inhibitory modulation of histamine-stimulated acid secretion in isolated rat parietal cells. The results also suggest that carbachol inhibition is Ca<sup>2+</sup> dependent, and is due to a modification of histamine H<sub>2</sub>-receptors or to an effect on histamine-stimulated cyclic nucleotide metabolism.

This work was supported by USPHS grant No. AM30517.

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## 29 Characteristics of muscarinic receptor M<sub>2</sub> subtype on canine gastric smooth muscle S. M. Collins

Intestinal Disease Research Unit, McMaster University, Hamilton, Ontario, Canada.

In the present study we have identified the muscarinic receptor subtype on gastric smooth muscle using selective antagonists, and have examined its regulation by guanine nucleotides.

Smooth muscle cells were isolated from the circular layer of the canine gastric corpus by collagenase digestion and suspended in a Krebs-HEPES buffer containing 1.2 mM MgCl<sub>2</sub>. Contraction was measured by image-splitting micrometry after fixing with acrolein, and binding studies were performed in the same buffer using [<sup>3</sup>H]N-methylscopolamine (NMS) in the presence or absence of atropine (10 μM).

The cells contracted in a concentration-dependent manner following a 30 s exposure to 0.1 μM-0.1 mM oxotremorine. Binding of [<sup>3</sup>H]NMS was rapid, reversible, stereospecific, pharmacologically selective and saturable, yielding a K<sub>D</sub> value for NMS of 106 pM. There was homogeneity of antagonist binding as illustrated by Hill coefficients close to unity for atropine (0.96), 4 diphenylacetoxy-N-methyl piperidine methiodide (4-DAMP; 0.95) and pirenzepine (0.94). In contrast, the binding of agonists was heterogeneous as illustrated by the shallow curve for inhibition of NMS binding by oxotremorine in

concentrations ranging from 0.6 nM to 0.1  $\mu$ M, yielding a Hill coefficient of 0.32. There was a 140-fold higher affinity for 4-DAMP than for pirenzepine supporting an  $M_2$  classification of the muscarinic receptor on this tissue. Incubation of the cells with guanylyl-5'-yl-imidodiphosphate (GppNHp) did not shift the curve for inhibition of NMS binding by oxotremorine or atropine. Similarly, no effect of GppNHp was observed on the abilities of oxotremorine and atropine to inhibit [ $^3$ H]NMS binding to smooth muscle plasma membranes in  $Mg^{2+}$  free and low ionic strength media (10 mM Tris).

These results indicate that the high affinity muscarinic receptor on smooth muscle from the canine stomach is of the  $M_2$  subtype, with a high affinity for 4-DAMP and a low affinity for pirenzepine. In contrast to the  $M_2$  receptor on cardiac muscle, this receptor does not appear to be regulated by guanine nucleotides and this distinction may serve as a basis for further classification of this receptor subtype.

Supported by MRC Canada.

### 30 Telenzepine is more than 25-times more potent than pirenzepine – a dose-response and comparative secretory study in man U. Voderholzer, V. Londong, W. Londong, A. Meierl

Chirurgia und Medizinische Kliniken Innenstadt, University of Munich, Munich, FRG.

Telenzepine, an analogue of pirenzepine, seems to have a comparable selectivity, but a higher affinity to muscarinic receptor sites than pirenzepine – according to *in-vitro* and *in-vivo* studies in animals.

It was the purpose of this placebo controlled, double-blind, and randomized study to investigate the dose-response relationship of 2, 3, and 5 mg telenzepine p.o. and to compare their effects with that of 50 mg pirenzepine p.o. on peptone-stimulated gastric acid secretion, synchronously measured spontaneous salivation, and gastrin release in 10 healthy male subjects (median age: 25 years). Drug serum concentrations, peripheral pulse rates, and near point vision were monitored.  $p=0.01$  was considered to be significant.

Peptone-stimulated acid output/3 h ( $58 \pm 6$  mmol  $H^+$  for placebo, given as  $\bar{x} \pm SEM$ ) was significantly and dose-dependently inhibited by telenzepine (2 mg:  $31 \pm 5$ , 3 mg:  $23 \pm 5$ , 5 mg:  $21 \pm 4$  mmol  $H^+$ ); 3 and 5 mg telenzepine were significantly stronger than pirenzepine ( $37 \pm 8$  mmol  $H^+$ ). Basal and peptone-stimulated gastrin was unaffected. Percentage inhibition of salivation by telenzepine (2 mg: 43%, 3 mg: 39%, 5 mg: 76%) was significant versus placebo; the effect of pirenzepine was only moderate (13%). In 6 subjects there was a significant correlation of percentage inhibition of acid and salivary secretion. Salivary outputs of  $Na^+$ ,  $K^+$ ,  $Ca^{2+}$ , and amylase were effectively reduced by telenzepine. Near point vision was not altered by either drug. Pulse rates were significantly lowered by pirenzepine. Complaints of dry mouth were more often with 5 mg telenzepine.

Telenzepine proved to be a far more potent gastric antisecretagogue than pirenzepine, under the conditions

tested. Its closely related inhibition of salivation may limit its clinical use.

### 31 Interactions of pirenzepine and ranitidine in man – pharmacodynamic secretory studies W. Londong, G. Bozler, H. Eberl, R. Gugler, V. Londong, G. Pösch

Klinik Innenstadt, University of Munich, Munich, FRG, Institut für Pharmakodynamik, University of Graz, Austria, and Medizinische Klinik, University of Bonn, FRG.

As was shown in previous investigations, combined application of the  $H_2$ -blockers cimetidine or ranitidine and the antimuscarinic drug pirenzepine suppresses meal-stimulated gastric acid secretion and prevents rebleeding from gastroduodenal ulcers significantly more effectively than single drug treatment (Londong *et al.* 1980, 1981, 1982). Whether these effects are additive or synergistic, is not known.

It was the aim of these placebo-controlled, double-blind, and randomized studies to investigate the mode of interaction using a new approach (Pösch 1982) in which equieffective doses have to be defined at first and reduced dose-combinations tested thereafter. In 10 healthy subjects we measured peptone-stimulated gastric acid secretion for 3 h. Plasma concentrations of pirenzepine and ranitidine were estimated by radioimmunoassay and HPLC, respectively.

In the first series of experiments, 50 and 100 mg pirenzepine reduced acid output by 45 and 71%, respectively, and 50 and 100 mg ranitidine by 78 and 98%, respectively. Thus, 100 mg pirenzepine and 50 mg ranitidine proved to be equieffective. Percentage inhibition and AUC correlated significantly. In the second series, reduced dose-combinations suppressed acid-output significantly stronger than equieffective doses alone (92% by 25 mg pirenzepine + 37.5 mg ranitidine; 94% by 50 mg pirenzepine + 25 mg ranitidine; 91% by 75 mg pirenzepine + 12.5 mg ranitidine). An interaction on the plasma level site was excluded.

Our results support the assumption that the interaction of pirenzepine and ranitidine can be characterized as a synergistic or potentiating effect which should be applied clinically in definable indications.

### 32 Peptidergic inhibition of cholinergic drive via $M_1$ muscarinic neural receptors in canine small intestine *in vivo* J. E. T. Fox, E. E. Daniel and T. J. McDonald

Program for Study of Control of Smooth Muscle Function, McMaster University, Hamilton, Ontario and Department of Medicine, University of Western Ontario, London, Ontario, Canada.

Release of acetylcholine to muscarinic receptors on smooth muscle is a major excitatory mechanism in the gastrointestinal tract both *in vivo* and *in vitro*. However, acetylcholine administered by close i.a. injection to the

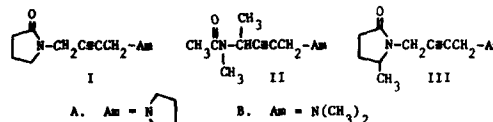
canine small intestine during sodium pentobarbital anaesthesia produces excitation followed by inhibition of phasic, cholinergic nerve driven activity is present in the segment. The dose of acetylcholine producing inhibition was not increased by hexamethonium, reserpine, or naloxone treatment but was eliminated by atropine or tetrodotoxin treatment and increased by pirenzepine treatment. Thus acetylcholine may produce inhibition by activating muscarinic ( $M_1$ ) receptors on nerves but not nicotinic, adrenergic, or opioid receptors. The putative  $M_1$  agonist McN-A-343 (4-[*m*-chlorophenylcarbamoyloxy]-2-butynyl-trimethylammonium chloride) produced only inhibition of cholinergic nerve driven phasic activity. Two natural peptides which act to produce inhibition via nerves (i.e. response is tetrodotoxin-sensitive), substance P, and Gastrin releasing peptide (GRP)/bombesin appear to activate these muscarinic  $M_1$  receptors, since the inhibitory responses are atropine and pirenzepine-sensitive but hexamethonium, reserpine, and naloxone-insensitive. The substance P receptor would appear to be SPP in nature since the order of sensitivity is physalaemin > SP >  $\alpha$  neurokinin > kassinin = eliodosin = neuromedin K and requires the substance P sequence (7-11) to produce an effect. When strips from similar segments of small intestine are studied *in vitro*, we have been unable to demonstrate the presence of the inhibitory receptor (i.e. inhibition of field-stimulated responses) by carbamylcholine, McN-A-343, or substance P. Only a direct smooth muscle excitation is evident. Thus the neural cholinergic inhibitory pathway activated by peptides appears to require an intact segment of small intestine to be demonstrated. Functionally this pathway seems to be able to regulate contractile activity as acetylcholine released in the vicinity of nerves by peptides would inhibit further acetylcholine release and 'brake' excessive contractions.

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### 33 Selectivity of oxotremorine analogs based on differential efficacy B. Ringdahl, R. Amstutz and D. J. Jenden

Department of Pharmacology, UCLA School of Medicine, Los Angeles, CA 90024, USA.

ED<sub>50</sub> values, affinity constants and relative efficacies of IA, IB, IIA, IIB, IIIA and IIIB at muscarinic receptors in the guinea pig ileum were estimated according to Furchgott. IIIA was a competitive antagonist. Agonist potencies decreased in the order: IA > IB > IIA > IIB > IIB. The rank order of affinities (IIIA > IIA > IA > IIB > IIB > IB) was different from that of efficacies (IB > IA > IIB > IIIA > IIA > IIIA). All compounds produced salivation in mice and sialagogic activity paralleled spasmogenic activity on the ileum. Only IA, IB and IIB caused tremor in mice. The enantiomers of IIA, IIIA and IIIB antagonized oxotremorine-induced tremor. Tremorolytic potency was correlated with affinity at ileal receptors. All compounds except IIIA produced analgesia (ED<sub>50</sub>: IA=0.06, IB=0.4, R-IIA=0.6, S-IIA=16, R-IIB=0.9, S-IIB=24, S-IIIB=0.8 and R-IIIB=10  $\mu$ mol kg<sup>-1</sup> i.p.) and hypothermia in mice. Analgesic potency



was correlated with spasmogenic and sialagogic activity. Thus IIA and IIIB discriminated between central muscarinic effects and also were more potent in stimulating post- than pre-synaptic ileal muscarinic receptors. IIIA separated spasmogenic and sialagogic effects. In contrast, more efficacious agonists (IA, IB and IIB) showed no such differentiation. This apparent selectivity seems to be related to differences in receptor reserve at different sites of action rather than to differences between receptors. The almost identical enantiomeric potency ratios in all tests support the idea of receptor similarity as do the correlations between *in-vitro* and *in-vivo* effects. These correlations also rule out distributional factors as a major contributor to the observed selectivity. Our results suggest that agonists of low efficacy may exert selectivity which is based on regional differences in receptor density and/or coupling processes.

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### 34 Characterization of muscarinic receptor subtypes involved in vagally-induced bronchoconstriction Marilyn Halonen, John W. Bloom and Henry Yamamura

Division of Respiratory Sciences and Department of Pharmacology, University of Arizona, Tucson, AZ 85724, USA.

The major excitatory pathway of airway regulation is thought to be via cholinergic neurons in the vagus nerve. Alterations in that regulation may contribute to the bronchial hyper-reactivity of asthma and other airway obstructive diseases. Stimulation of the peripheral ends of the transected cervical vagi induces bronchoconstriction and cardiac slowing in animals. Atropine inhibition demonstrates the dependence of these responses on cholinergic receptors. We compared the antagonist activities of atropine with those of pirenzepine, a selective muscarinic antagonist. Vagal stimulation (40 Hz, 0.3 ms, 10 V) induced consistent, reversible bronchoconstriction as reflected in an increase in total pulmonary resistance (% increase in  $R_L$  =  $180 \pm 23$ ) and an almost complete stopping of the heart rate (% decrease in heart rate =  $98 \pm 4$ ). The response was reproducible upon 12 repeat stimulations. Six rabbits were infused intravenously with increasing concentrations of atropine in half-log increments, and the per cent inhibition of the changes in  $R_L$  and heart rate were determined for each concentration. (The rabbits were anesthetized, paralysed and mechanically ventilated.) Six additional rabbits were studied identically but with pirenzepine infusion. IC<sub>50</sub>s for atropine inhibition of the increase in  $R_L$  was 0.6 nmol kg<sup>-1</sup> min<sup>-1</sup> and for inhibition of the decrease in HR was 1.6 nmol kg<sup>-1</sup> min<sup>-1</sup>. IC<sub>50</sub>s for pirenzepine inhibition

were 10.1 and 420 nmol kg<sup>-1</sup> min<sup>-1</sup> for the increase in  $R_i$  and decrease in heart rate, respectively. Thus, we have demonstrated that pirenzepine is a potent, selective antagonist for vagally induced bronchoconstriction, being only 17-fold less potent than atropine, whereas pirenzepine is 260-fold less potent than atropine in the vagal pathway for cardiac slowing. These results suggest that selective muscarinic antagonists such as pirenzepine may have significant therapeutic potential in airways obstructive diseases.

Supported in part by NIH grant HL 31219.

**35** McN-A-343: a selective agonist at  $M_1$  receptors in terms of potency but not affinity *R. M. Eglen, A. D. Michel and R. L. Whiting*

Syntex Research Centre, Edinburgh EH14 4AS, Scotland, UK.

McN-A-343 (4-[*m*-chlorophenylcarbamoyloxy]-2-butylnyl-trimethylammonium chloride) has been proposed as an  $M_1$  muscarinic receptor agonist since it stimulates muscarinic receptors present on the sympathetic ganglia and has little effect at muscarinic receptors present on the ileum. However, in addition to receptor heterogeneity, the presence or absence of a receptor reserve may also influence the production of a response to a given agonist, particularly agonists of low efficacy. The aim of the study was to determine the affinity and potency of McN-A-343 at a range of both  $M_1$  and  $M_2$  muscarinic receptors.

Taenia caeci, ileum and atria (spontaneously beating) were removed from guinea-pigs. Agonist potencies to both carbachol and McN-A-343 were then determined. In cases where McN-A-343 was employed as an antagonist, carbachol was used as an agonist. Ligand binding experiments were undertaken using membranes from rat cerebral cortex and myocardium.

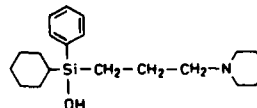
McN-A-343 elicited a marked pressor action in the pithed rat (10, 30 and 100 mg kg<sup>-1</sup> elicited 7%, 68% and 154% rise in mean arterial blood pressure respectively) and also a contractile response in the isolated taenia caeci ( $EC_{50} = 7.3 \times 10^{-6}$  M). No response was observed in the ileum or atria. In these two latter preparations the compound acted as a competitive antagonist (ileum  $pA_2 = 4.46$ ; atria  $pA_2 = 4.75$ ). These affinity values were similar to those observed in ligand binding studies using cortical and atrial membranes (cortex  $K_i = 9.0 \times 10^{-6}$  M; myocardium  $K_i = 6.0 \times 10^{-6}$  M). Pirenzepine antagonized the contractile response of the taenia to McN-A-343 with an affinity consistent with  $M_2$  receptor activation (6.82) whereas it antagonized that in the pithed rat with a potency consistent with  $M_1$  stimulation.

In summary, the affinity of McN-A-343 at a range of muscarinic receptors was very similar but its potency at  $M_1$  receptors in the pithed rat was very great in comparison to  $M_2$  receptors present in the ileum and atria. A response was observed in the taenia but this was due to  $M_2$  stimulation. Therefore, the response to McN-A-343 alone is not evidence itself of the presence of  $M_1$  receptors, since the receptor reserve influences the production of a response.

**36** Hexahydrosiladifenidol: a selective antagonist at muscarinic  $M_2$  receptor subtypes *G. Lambrecht, U. Moser, J. Wess, J. Riotte, H. Fuder, H. Kilbinger, H. Müller, H. Linoh, R. Tacke, H. Zilch and E. Mutschler*

Department of Pharmacology, University of Frankfurt, D-6000 Frankfurt M-70, and Department of Pharmacology, University of Mainz, D-6500 Mainz, FRG.

The differentiation of two types of muscarinic receptor, designated  $M_1$  and  $M_2$  was suggested on the basis of experiments with the agonist McN-A-343 (4-[*m*-chlorophenylcarbamoyloxy]-2-butylnyl-trimethylammonium chloride) and the antagonist pirenzepine. While it appears that the  $M_1$  receptors are homogeneous, we found indications that the  $M_2$  receptors are heterogeneous. Studies on anti-muscarinic agents of the procyclidine- and difenidol-type have shown that substitution of the central C-atom ( $R_3$ -C-OH) by silicon leads to drugs with increased anti-muscarinic potency and increased selectivity for subtypes of muscarinic  $M_2$  receptors. In peripheral neurons and effector organs this observed selectivity is most pronounced with hexahydrosiladifenidol. The antimuscarinic effects of hexahydrosiladifenidol on sympathetic ganglionic muscarinic receptors, and on receptors of the vascular endothelium were studied in pithed and anaesthetized rats. *In-vitro* experiments were carried out at postsynaptic muscarinic receptors in atrial pacemaker and myocardium cells, in the ileum and in the urinary bladder of the guinea-pig and rat, respectively. Additionally, affinities for presynaptic muscarinic receptors were determined in the guinea-pig ileum and rat heart.



Hexahydrosiladifenidol shows a 15- to 30-fold higher antimuscarinic potency at muscarinic receptors of the ileum and urinary bladder than at those of the other preparations. As far as heart and ileum are concerned, hexahydrosiladifenidol does not discriminate between pre- and post-synaptic muscarinic receptors within the same organ. Thus, hexahydrosiladifenidol seems to be a tool for classification of muscarinic  $M_2$  receptor subtypes in peripheral neurons and effector organs, and a lead to design more selective drugs for treatment of disorders in the function of the gastrointestinal-tract and the urinary bladder, respectively.

**37** The efficiency of presynaptic receptor-effector coupling in the muscarinic inhibition of norepinephrine release from rat hearts *H. Fuder and H. Müller*

Department of Pharmacology, University of Mainz, FRG.

The potency of agonists as reflected by the concentration of half-maximum response,  $EC_{50}$ , is determined by the

affinity of the agonist for the receptor and the efficiency of coupling processes between receptor activation and pharmacological response<sup>1</sup> (efficacy). Only little is known about affinity constants ( $K_A$ , dissociation constant of the agonist-receptor complex) and relative efficacies (estimated from the fractional receptor occupation or the extent of spare receptors at maximum response) of muscarinic agonists in presynaptic effector systems.

The aim of the present study was to determine  $K_A$  and efficacies of acetylcholine and arecaidine propargyl ester (APE) in inhibiting the sympathetic nerve stimulation-evoked [<sup>3</sup>H]norepinephrine release from the rat isolated perfused heart. The methods used have been described before<sup>2,3</sup>. In the presence of physostigmine, corticosterone, desipramine, phentolamine, and propranolol, the  $EC_{50}$  of acetylcholine (40 nM, causing 50% inhibition of transmitter overflow evoked by 10 electrical pulses at 10 Hz) was 35-times lower than  $K_A$  (1.4, 0.9–2.3  $\mu$ M; geometric mean, fiducial limit;  $n=10$ ), indicating a spare receptor capacity of 97.5% of total presynaptic muscarinic receptor population at  $EC_{50}$ . The  $EC_{50}$  of APE (50 nM) was 66-times lower than  $K_A$  (3.3, 2.3–4.8  $\mu$ M;  $n=6$ ) indicating that occupation of 1.6% of total receptors resulted in halfmaximal effect. A 95% inhibition of the [<sup>3</sup>H]norepinephrine release was observed when either agonist occupied only 5–10% of the receptors. In contrast, the  $K_A$  of pilocarpine (10  $\mu$ M) was only slightly higher than  $EC_{50}$  (6.2  $\mu$ M), and no receptor reserve was detected for pilocarpine.<sup>2</sup>

The results show that the presynaptic muscarinic effector system of rat heart adrenergic nerves behaves as predicted by receptor theory. The presynaptic agonist potency depends not only on the affinity, but also on the efficacy. According to agonist  $K_A$  values investigated so far, presynaptic and postsynaptic<sup>1</sup> muscarinic receptors cannot be distinguished.

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### 38 Muscarinic receptor subtypes involved in the release of acetylcholine from the mouse cerebrum and the furan analogs of muscarine B. V. Rama Sastry and N. Jaiswal

Vanderbilt University School of Medicine, Nashville, TN 37232, USA.

Previous studies have indicated that two feedback mechanisms, one positive and the other negative, regulate the rate of acetylcholine release. Muscarinic receptors are components of both of these feedback mechanisms (*Adv. Biosci.*, 38: 165–172, 1982; *Pharmacology* 26: 61–72, 1983). In order to evaluate the subtypes of muscarinic receptors involved in these feedback mechanisms for the release of acetylcholine in the mouse cerebrum, the effects of muscarine and a series of furan analogs of muscarine, 5-methylfurfuryltrimethylammonium (5-MFT), 5-chloro-

methylfurfuryltrimethylammonium (5-CMFT), 5-hydroxy-methylfurfuryltrimethylammonium (5-HMFT) and 5-methoxyfurfuryltrimethylammonium (5-MOFT), were studied on the release of acetylcholine from the mouse cerebral slices. Mouse cerebral slices were incubated in a modified Krebs Ringer buffer containing (methyl-<sup>3</sup>H)choline (0.1 mM; 0.25  $\mu$ Ci ml<sup>-1</sup>) for 60 min. They were filtered, washed and transferred to a microbath set up for superfusion with the above buffer containing hemicholinium-3 (10  $\mu$ M). The release of [<sup>3</sup>H]acetylcholine into the superfusate was measured as a function of time. Muscarine (0.48–480 nM), 5-MFT (13–1300 nM) and 5-CMFT (10–1000  $\mu$ M) decreased spontaneous release of acetylcholine by about 15–20% of the control effect. The effect of these agents increased to 40–60% of control by increasing concentrations of the agonists. 5-HMFT (1.9 nM) and 5-MOFT (1.9 nM) decreased the spontaneous release of acetylcholine by about 40%. However, this effect decreased to about 15% of control by increasing the concentrations of 5-HMFT and 5-MOFT (to 190  $\mu$ M). The effects of these agents on the evoked release of acetylcholine were similar. The effect of 5-MFT on acetylcholine release was antagonized by atropine (1  $\mu$ M) but not naloxone (55 nM). The effect of 5-HMFT on acetylcholine release was antagonized by scopolamine (10 nM) and naloxone. Muscarine, 5-MFT and 5-CMFT were considerably more potent than 5-HMFT and 5-MOFT on the muscarinic receptors of the smooth muscle. These observations indicate that muscarine and furan analogs of muscarine can be divided into two groups which activate two different types of muscarinic receptors in the mouse cerebrum.

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### 39 Autoradiography of $M_1$ and $M_2$ muscarinic binding in the striatum M. A. Nastuk and A. M. Graybiel

Department of Psychology and Whitaker College, MIT, Cambridge, MA 02139, USA.

In a previous autoradiographic study with [<sup>3</sup>H]propylbenzylcholine mustard ([<sup>3</sup>H]PrB) we found that muscarinic ligand binding is highly heterogeneous during striatal development, with patches of dense binding appearing in a sparser background. In several species including the human, the patches of dense [<sup>3</sup>H]PrB binding coincided with islands of nigrostriatal dopamine innervation. This suggested an ontogenetic linkage between muscarinic binding and dopamine-containing striatal afferents (Nastuk & Graybiel '83, '85). In the mature striatum, the sites of dopamine islands become the 'striosomes', macroscopic compartments with respect to which many striatal properties are organized. With [<sup>3</sup>H]PrB we found only faint signs of heterogeneity in the mature striatum (adult cat) though a few patches of heightened binding density were seen, and matched striosomes.

To learn whether overall muscarinic binding had become uniform or whether heterogeneity persisted for

one subtype but was masked by homogeneity of another subtype, we performed autoradiography in conditions favoring the labeling of  $M_1$  or  $M_2$  muscarinic subtypes in striatal tissue from adult cats. To identify  $M_1$  and  $M_2$  sites, binding conditions were chosen according to protocols of Hammer *et al.* '80 (and unpublished observations) and Potter *et al.* '84: sites labeled with [ $^3$ H]pirenzepine ([ $^3$ H]PZ; 10 nM) were termed  $M_1$  and sites bound with [ $^3$ H]-*N*-methylscopolamine ([ $^3$ H]NMS; 0.3 nM) in the presence of 100 nM pirenzepine were termed  $M_2$ . Binding assays of striatal tissue yielded  $K_D$ 's of 7.6 nM ([ $^3$ H]pirenzepine) and 0.42 nM ([ $^3$ H]NMS) and showed saturable specific binding and very low levels of nonspecific binding.

In the mature striatum, sites autoradiographically labeled under  $M_1$  conditions were in patches having particularly high binding density and matching striosomes. Putative  $M_2$  binding appeared virtually homogeneous. Fetal brains were also studied (E40-E58); both  $M_1$  and  $M_2$  sites were clustered, and the patches of binding corresponded to dopamine islands. Perinatally, both  $M_1$  and  $M_2$  distributions gradually changed.  $M_2$  binding became uniform while  $M_1$  binding became less crisply patchy before becoming resolved into the adult distribution.

The finding of elevated  $M_1$  but not  $M_2$  binding in striosomes suggests that aspects of striatal cholinergic function are subject to compartmental organization and so are related to the heterogeneity of dopaminergic input to the striatum.

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#### 40 Direct autoradiographic determination of muscarinic receptor sub-type distribution in the rat brain: relative binding patterns in cholinergic nuclei and projection areas E. Horvath, J. Traber and D. G. Spencer, Jr.

Neurobiology Department, Troponwerke, Neurather Ring 1, 5000 Cologne 80, FRG.

The autoradiographic distribution of [ $^3$ H]oxotremorine-M and [ $^3$ H]pirenzepine binding to rat brain  $M_1$  and  $M_2$  receptors, respectively, was studied in order to evaluate the hypothesis that  $M_2$  receptors are good markers for cholinergic cell bodies and tracts, as opposed to  $M_1$  receptors which appear to label post-synaptic fields.<sup>1</sup> The binding of 1 nM [ $^3$ H]oxotremorine-M to brain slices was highly specific and saturable. Structurally unrelated muscarinic receptor ligands such as scopolamine, carbachol, and arecoline produced complete competition at 1, 10, and 100  $\mu$ M, respectively. Regional distribution of  $M_2$  binding was quite different from that of  $M_1$  binding (labelled by 1 nM [ $^3$ H]pirenzepine):  $M_2$  receptors were found in brain-stem cholinergic nuclei (e.g., cranial nerve nuclei, parabrachial nuclei, dorsal and latero-dorsal tegmental nuclei, trapezoid body nuclei, raphe nuclei, central gray, interpeduncular nuclei, and pontine nuclei, inferior and superior colliculus, several thalamic areas (e.g., ant. pretectal area and anterodorsal, anteromedial,

anteroventral, lateral posterior, posterior paraventricular, reticular, reuniens, and rhomboid nuclei), hypothalamic regions, forebrain cholinergic nuclei (e.g., medial septum, diagonal band horiz. and vert. limb nuclei, nucleus basalis, substantia innominata), caudate-putamen, CA2 and CA3 of hippocampus, amygdala (primarily the basolateral nuclei), olfactory bulbs and tubercle, cingulate gyrus, perirhinal cortex, and layers III and V of cerebral cortex;  $M_1$  receptors were found only in telencephalic structures such as layers I and II of the cerebral cortex (frontal, parietal, and striate cortex), caudate-putamen, nucleus accumbens, olfactory tubercle, CA1 and dentate gyrus of the hippocampal formation, and basolateral nuclei of amygdala. This pattern of  $M_1$  and  $M_2$  binding supports the idea that  $M_2$  receptors are closely associated with cholinergic cell nuclei and projection areas. However,  $M_1$  receptors can only be detected in telencephalic cholinergic terminal regions; only  $M_2$  receptors were found in the thalamic and collicular terminal zones. Scatchard analysis of [ $^3$ H]oxotremorine-M slice binding in the inferior colliculus revealed one high-affinity site ( $r = -0.95$ ), with a  $K_D$  of 1.88 nM and a  $B_{max}$  of 1.42 pmol  $mg^{-1}$  protein. 1 mM *N*-ethylmaleimide reduced the affinity of  $M_2$  receptors for [ $^3$ H]oxotremorine-M without altering their regional distribution in brain;  $M_1$  receptor distribution and affinity for [ $^3$ H]pirenzepine were unaffected by this treatment. Taken together, these data strongly support the hypothesis that  $M_1$  and  $M_2$  receptors are not simply intraconvertible states of a single receptor protein, but may be anatomically and functionally distinct muscarinic receptor sub-types.

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#### 41 Distribution of $M_1$ and $M_2$ muscarinic acetylcholine receptors on cortical neurons and thalamic afferents B. A. Vogt, E. Townes-Anderson and D. L. Burns

Boston University School of Medicine, Boston, MA 02118, and Cornell University Medical College, New York, NY 10021, USA.

There are approximately equal proportions of  $M_1$  and  $M_2$  muscarinic receptors in the cerebral cortex and classical muscarinic antagonists like propylbenzylcholine mustard (PrBCM) bind to both of them. In a recent study of cingulate cortex it was observed that PrBCM binding is associated with anterior thalamic axons and cortical neurons. The present analysis considers two questions. Are the  $M_1$  and  $M_2$  receptors associated with thalamic and cortical neurons differentially? What is the distribution of muscarinic receptors on isolated cortical cells?

Unilateral ablations were placed in rats in the anterior thalamic nuclei (ATN) or directly in cortex with the neurotoxin ibotenic acid. Following a two week post-operative survival, blocks from normal and experimental

cortices were sectioned with a cryostat. To dissociate neurons, brains were removed from ether anesthetized rats and the cortex cut into 0.2 mm thick sections which were incubated in papain, triturated and the cells placed on concanavalin A coated slides. Cryostat sections or cells were incubated in [ $^3$ H]PrBCM (2.4 nM) or [ $^3$ H]-pirenzepine (15 nM, selective  $M_1$  antagonist). Some sections were co-incubated in atropine (1  $\mu$ M) to assess nonspecific binding.

Specific binding of PrBCM is not uniform with peaks in laminae Ia and IV. Ablations of the ATN reduce this binding by 30% and 15%, respectively. In contrast, pirenzepine binding is essentially homogeneous across laminae and is not altered by ATN lesions.

Ibotenic acid ablations destroy cortical neurons but leave afferent axons and microvasculature intact. In these cases specific PrBCM binding is reduced by 70%, and that of pirenzepine by 80%. Thus, the primary reduction in muscarinic binding is probably associated with the loss of  $M_1$  receptors on neurons.

After cortical dissociation and incubation in PrBCM binding is most pronounced on the secondary and tertiary dendrites of pyramidal neurons. There is less specific binding on the apical dendrites of these neurons and the dendrites of multipolar cells. Most astroglial binding appears to be nonspecific.

We propose a model of cholinergic architecture in which  $M_1$  receptors are located primarily on the dendrites of cortical neurons whereas the  $M_2$  subtype is located mainly on ATN axons.

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#### 42 Regional distribution of gallamine binding sites in the rat brain W. Hoss, M. Price, and W. S. Messer, Jr.

Center for Brain Research, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642, USA.

Recently, the antagonist gallamine has been shown to distinguish between muscarinic receptors in brain on the basis of affinity.<sup>1</sup> In the present study, the regional distribution of muscarinic receptors recognized by the antagonist gallamine was determined autoradiographically by the ability of gallamine to reduce the binding of [ $^3$ H]-quinuclidinyl benzilate (QNB) in rat brain slices. Rat brain slices were incubated in the presence of 0.2 nM [ $^3$ H]-QNB as well as with various concentrations of gallamine. Nonspecific binding of QNB was measured by incubation in excess atropine. The inhibition data obtained from indirect binding assays on whole slices indicated that gallamine distinguishes at least two sites with differing affinities ( $K_i = 0.6 \mu$ M,  $K_i = 10 \mu$ M). Following exposure of the sections to LKB tritium-sensitive Ultrafilm and development of the film, the regions of highest and lowest affinity for gallamine were qualitatively apparent by visual inspection of the autoradiograms. A number of regions in coronal sections at three different levels were compared by quantitative microdensitometry.

We report that gallamine possessed greater overall affinity for the diencephalon and brainstem than for the

forebrain as measured by the ability to inhibit the labeling of [ $^3$ H]-QNB. At lower concentrations of gallamine ( $1 \times 10^{-6}$  M), the cerebral cortex (20% inhibition), corpus striatum (15%) and hippocampus (5%) displayed lower affinity for gallamine, while thalamic (45%) and septal nuclei (55%) were regions of higher affinity for gallamine. Within the brainstem, the superior colliculus (67%) contained the greatest proportion of sites with high affinity for gallamine. Higher doses of gallamine ( $1 \times 10^{-5}$  M) inhibited [ $^3$ H]-QNB labeling more uniformly (70%). Compared to the binding of other muscarinic ligands, the binding profile of gallamine is opposite to that of the antagonist pirenzepine and similar to that of the agonist carbamylcholine, suggesting that gallamine is selective for  $M_2$  muscarinic receptors.

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#### 43 Muscarinic receptor density changes in rat hippocampal formation due to age, deafferentation and chronic blockade are greatest in subiculum R. Loy, M. W. Tayrien, J. E. Springer, S. L. Carlson and J. M. Ordry

Department of Neurobiology and Anatomy, University of Rochester, Rochester, N.Y. 14642 and Pennwalt Corp., Rochester, N.Y. 14623, USA.

The cholinergic innervation of the hippocampal formation (HF) arises mainly in the medial septum. Denervation does not upregulate muscarinic quinuclidinyl benzilate ([ $^3$ H]QNB) receptor binding in HF homogenates; however, [ $^3$ H]QNB binding does increase in HF homogenates in response to scopolamine treatment. We have used quantitative, regional receptor autoradiography to resolve this apparent discrepancy in the effects of cholinergic inactivation on muscarinic receptor binding in HF.

Adult female rats received unilateral lesions of the fimbria or 10 mg kg<sup>-1</sup> scopolamine daily. After 14 days rats were decapitated and 20  $\mu$ m frozen sections incubated in 1.3–2.0 nM [ $^3$ H]QNB with or without 1  $\mu$ M atropine, and exposed to LKB Ultrafilm. Autoradiographs were analysed using the ARIA program for the Nikon Magiscan. Specific [ $^3$ H]QNB binding decreases in the ipsilateral HF for at least 1.5 mm caudal to the lesion, but further caudally increases by 20%. The largest increases are in the dorsal subiculum (30%), CA3 (20%) and dentate gyrus (17%). In the most caudal sections binding again decreases in the ipsilateral HF. Chronic scopolamine treatment increases [ $^3$ H]QNB binding 25% in the whole HF at rostral levels, with the greatest increases being in the subiculum (46%) and CA1 (41%).

Cholinergic activity decreases with age in HF, although most studies find no change in muscarinic receptor binding in HF homogenates. Using autoradiography we

compared brains from 12- and 40-month-old rats. [<sup>3</sup>H]QNB binding in rostral HF does not differ in aged and young adult brains. In caudal HF, specific binding is higher in the subiculum (40%), CA1 (27%) and dentate gyrus (25%) in the aged brains.

These combined studies show that differences in cholinergic activity due to age, or produced by receptor blockade or denervation, result in complementary differences in muscarinic receptor binding, and that the most sensitive region of the HF is the subiculum.

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#### 44 Changes in muscarinic receptors after behavioral tolerance to pirenzepine and scopolamine W. S. Messer, Jr., W. Hoss, G. J. Thomas, and M. Price

Center for Brain Research, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642, USA.

Muscarinic antagonists, after injection into the hippocampus, impaired performance of rats on a representational task. Pirenzepine, an M<sub>1</sub> selective antagonist, produced representational memory deficits as measured by the percentage of correct choices in the non-matching to sample task. Scopolamine, a less selective muscarinic antagonist, caused increases in running times, preventing a definitive interpretation of the nature of the impairment. Pirenzepine displayed a higher affinity for the hippocampus and was more effective in producing a selective impairment of representational memory than scopolamine. The data indicated that cholinergic activity in the hippocampus was necessary for representational memory function. Tolerance to the effects of the muscarinic antagonists developed following initial exposures to the drugs.

Quantitative autoradiographic analysis of muscarinic receptors indicated that each ligand produced significant differences in the binding profile for muscarinic ligands when compared to saline-injected controls. Total [<sup>3</sup>H]-1-QNB (quinuclidinyl benzilate) binding was significantly increased in the cerebral cortex and cingulate cortex of pirenzepine-injected animals, but was unchanged in scopolamine-injected animals. Low concentrations of both scopolamine and pirenzepine were less effective in inhibiting QNB labeling in pirenzepine-injected animals as compared to saline-injected controls. Low concentrations of carbamylcholine were more effective in inhibiting [<sup>3</sup>H]-1-QNB labeling in the internal layer of the cerebral cortex, in the CA1 of the hippocampus, and in the pretectal area for scopolamine-injected animals as compared to saline-injected animals. High affinity agonist binding was not significantly different in pirenzepine-injected animals when compared to control animals.

Scopolamine and pirenzepine produced significant changes in the binding of muscarinic ligands that may be associated with the development of tolerance to each ligand. The changes associated with antagonist administration differed for each ligand and may indicate separate mechanisms of tolerance for each muscarinic antagonist.

#### 45 Blockade of cholinergic receptors by PrBCM in the rat cerebral cortex causes deficits in passive avoidance learning H. Yoshida, I. Fukuchi, M. Nakahiro and S. Uchida

Department of Pharmacology, Osaka University School of Medicine, Kita-ku, Osaka 530, Japan.

Effects of blockade of muscarinic acetylcholine receptors in the rat cerebral cortex on learning and memory assessed by performance of a step-through passive avoidance task were examined.

When initial trial for passive avoidance task was done at 24 h after bilateral injection of 9-90 µg propylbenzylcholine mustard (PrBCM) into frontal or parietal cortex and retention time in the second trial was examined at 24 h after the initial trial, rats showed shorter latency than those of control and amount of muscarinic acetylcholine receptor determined by [<sup>3</sup>H]quinuclidinyl benzilate decreased. But the injection into occipital cortex had no significant effect on the learning-memory process. Furthermore, the injection of 22.5 × 4 µg PrBCM into both frontal and parietal cortices completely impaired the process. Then in the following studies 22.5 × 4 µg PrBCM was injected into both cortices. When the initial trial was performed at 14 days after the injection the effect of PrBCM could not be observed indicating disappearance of the effect of PrBCM within 14 days. As the memory acquired by initial trial was maintained at least for 1 month in untreated rats, the effects of PrBCM on three phases (acquisition, retention and recall) tentatively assumed in learning-memory process were examined as follows: (1) initial trial was performed at 24 h after injection and the retention time in the second trial was examined at 14 days after the initial trial - in this case retention latencies were short; (2) when PrBCM was injected at 24 h after the initial trial and the retention time for the second trial was determined at 14 days after the injection, the retention latencies were not significantly different from that of control rats; and (3) PrBCM was injected at 24 h after the initial trial and the retention test was done at 24 h after the injection. Shortening of retention time was observed in this case. These results indicated that cholinergic function participated in both acquisition and recall phases but not in retention phase in the learning-memory process.

#### 46 Muscarinic cholinergic binding sites respond to acquisition and extinction of conditioned emotional response (CER) John D. Lane

Department of Pharmacology, Texas College of Osteopathic Medicine, Fort Worth, TX 76107, USA.

Rats were classically conditioned to associate a conditioned stimulus (CS) with footshock, so that on test day, CS presentation alone produced suppression of food-reinforced responding and collateral 'emotional' behaviors reminiscent of anxiety (conditioned emotional

response, CER). Previous studies<sup>1</sup> demonstrated that this paradigm resulted in total behavioral suppression and a 40% reduction in cortical muscarinic cholinergic (quinuclidinyl benzilate; QNB) binding sites (change in  $B_{max}$  and not  $K_D$ ). These experiments were extended to assess the role of repeated CS presentations (extinction) on binding. After CER conditioning, rats were subjected to 15 additional once daily trials of 60 min food-reinforced responding, during which the CS was presented continuously during the final 15 min, but footshock was never delivered. At two day intervals groups of rats were sacrificed and cortical QNB binding assessed. Control groups for pre-CS and for no-CS (up to 11 trials) were run to ensure that baseline binding parameters did not vary over the entire course of the experiment. Behavioral suppression extinguished within 13 trials. Cortical QNB binding (changes in  $B_{max}$  and not  $K_D$ ) followed a parallel time course and returned to within normal limits. Benzodiazepine binding was also assessed. At trial 1, binding was decreased 25% (change in  $B_{max}$  and not  $K_D$ ) but was still decreased 11% after 15 trials of extinction. The operant chamber may represent a pseudo-CS for the animals. These transient phenomena are consistent with other behavioral paradigms, and suggest that the cholinergic system mediates or responds to conditioning-emotion. The effects of cholinergic agonists and antagonists, and acetylcholinesterase inhibitors on the behavior and binding parameters are now being assessed.

Supported in part by MH-31835.

#### Reference

- 1 Lane et al. (1982) *Eur. J. Pharmacol.* 83, 183

#### 47 Chronic tabun injections result in altered cholinergic drug sensitivity John M. Carney and S. B. McMaster

Department of Pharmacology, Oklahoma Health Science Center, Oklahoma City, OK 73190, Neurotoxicology Branch, USAMRICD, Edgewood, MD, USA.

Rats were trained to respond under a multi-component operant schedule for food reward. A 10 min extinction period (all lights off and no available food reward) alternated with a 10 min period of available food reward (45 mg food pellet). The food pellet was available under a 30 response fixed ratio (FR-30) schedule of reinforcement. Dose-effect curves were determined using cumulative doses. Injections were given at the beginning of each extinction component. The session ended at the end of the fourth food reward component. Cumulative dose-effect curves were determined for atropine and oxotremorine before and after chronic tabun injections. Atropine produced dose-related decreases in FR responding. The  $ED_{50}$  for atropine was approximately 1 mg  $kg^{-1}$  (i.p.). The  $ED_{50}$  for oxotremorine-induced decreases was 0.056 mg  $kg^{-1}$  (i.p.). Tabun also produced decreases in responding with an initial  $ED_{50}$  of 0.015 mg  $kg^{-1}$  (s.c.). A cumulative total dose of 0.032 mg  $kg^{-1}$  (s.c.) was injected every third day. The cumulative dose

series used was 0.0032, 0.01, 0.0178 and 0.032 mg  $kg^{-1}$  (s.c.). By the third cumulative injection day the 0.032 mg  $kg^{-1}$  tabun dose had no significant effect on behavior. The extent of tolerance was determined on the sixth injection day (18 days after the start of chronic dosing). The tabun dose-effect curve was shifted to the right (tolerance) 30-fold. Redetermination of the atropine and oxotremorine curves demonstrated a significant change in cholinergic sensitivity. The atropine curve was shifted to the left approximately ten-fold and the oxotremorine curve was shifted to the right about five-fold. These results support the hypothesis that at least part of tabun tolerance development is due to down-regulation or loss of cholinergic receptor system.

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#### 48 An animal model of human-type memory loss based on aging, drug, and lesion studies with the rat J. M. Ordy, R. C. Griffith, J. C. Blosser, G. Thomas and W. Dunlop

Pennwalt Corp., Rochester, NY 14623, University of Rochester, NY 14624, and Tulane University, New Orleans, LA 70118, USA.

Loss of recent memory represents an inevitable manifestation of aging, particularly of Alzheimer's disease. These age and disease related memory impairments have been related to degeneration and/or loss of neurotransmitter-specific neurons in the basal forebrain, and the septo-hippocampal entorhinal circuit. Research strategies for the development of effective drug treatments for human type memory loss in animal models have focused on the essential criteria for measuring trial-specific working memory for correlation with neural changes in neurotransmitter-specific memory circuits produced by aging, drugs and lesions. The goals of this research program were to develop a valid test of trial-specific working memory with concomitant measures of motivation and neuromuscular performance for the rat in a T-Maze. Specific aims were to examine the effects of: (1) age; (2) basal forebrain, septal, amygdala lesions; and (3) physostigmine, scopolamine, and piracetam on memory, motivation, and motor performance of young, middle aged, and old rats. Aging significantly impaired working memory, motivation and motor performance. Memory of septally lesioned rats was significantly more impaired than that of basal forebrain, or amygdala lesioned rats. Physostigmine improved, whereas scopolamine impaired memory. Physostigmine also blocked scopolamine impairment of memory. The 'nootropic' drug piracetam did not improve memory, nor block scopolamine impairment of memory. Neurochemical evaluations revealed significant age related decreases in cholineacetyltransferase (CAT) activity in the basal forebrain, medial septum and dorsal hippocampus. Acetylcholinesterase (AChE) enzyme activity did not decrease with age in these regions; however CAT/AChE ratios changed significantly, indicating imbalances in cholinergic enzyme activity with age. The findings provide support for the

view that clarification of age, lesion, and drug effects on neural memory circuits in animal models represents a very important step in strategies for the development of effective drug treatments for memory loss in Alzheimer's disease.

**49 Studies of selective cholinergic biochemical markers in the anterior cerebral cortex of a proposed rat model of Alzheimer's disease** *Mark Watson, Thomas W. Vickroy, Hans C. Fibiger, William R. Roeske and Henry I. Yamamura*

*University of Arizona School of Medicine, Tucson, AZ 85724 USA, and Division of Neurological Sciences, University of British Columbia, Vancouver, BC, Canada V6T 1W5.*

The bilateral ibotenate-induced lesion of the nucleus basalis magnocellularis (Meynert in humans) has recently been suggested as an effective model for the study of Alzheimer's disease on the basis of behavioral, biochemical and pathological data. Thus, the relationship of choline acetyltransferase (CAT) activity and high affinity binding of the potent and selective sodium-dependent choline uptake inhibitor [ $^3$ H]hemicholinium-3 ([ $^3$ H]HC-3) to high affinity binding of the muscarinic agonist [ $^3$ H](+)-cismethyldioxolane ([ $^3$ H](+)-CD), the putative  $M_1$  selective antagonist [ $^3$ H]pirenzepine ([ $^3$ H]PZ) and the classical antagonist [ $^3$ H](−)-quinuclidinyl benzilate ([ $^3$ H](−)-QNB) in homogenates of the rat neocortex was studied. CAT activity was 42% lower in rats with ibotenate-induced lesions of the nucleus basalis magnocellularis (nbm) when compared to controls, and [ $^3$ H]HC-3 binding was similarly reduced by 44%. However, equilibrium dissociation constants ( $K_D$  values) for [ $^3$ H]HC-3 (0.8–1.0 nM), [ $^3$ H](−)-QNB (11–24 pM), [ $^3$ H]PZ (4.0–4.3 nM) and [ $^3$ H](+)-CD (2.1–2.9 nM) were each unchanged. Mean  $B_{max}$  values (total binding site densities) for [ $^3$ H](+)-CD were significantly altered in both hemispheres of the anterior cerebral cortex, showing a 25% reduction in the number of sites which display the highest affinity conformation for this potent muscarinic agonist. The decreased CAT activity and [ $^3$ H]HC-3 binding after nbm lesions were associated with only slight reductions in putative  $M_1$  muscarinic binding site density (14%) and [ $^3$ H](−)-QNB binding site density (13%). Thus, it appears that while [ $^3$ H]PZ and [ $^3$ H](−)-QNB label predominantly post-synaptic muscarinic binding sites, a significant number of sites labeled by [ $^3$ H](+)-CD may be associated

with presynaptic cholinergic nerve terminals. These data suggest that cholinergic input differentially regulates the drug binding sites of anterior cerebral cortical muscarinic receptors, exerting a substantial effect upon the highest affinity conformational state for agonists.

**50 Changes in muscarinic receptors in normal aging and dementia disorders** *A. Nordberg, A. Adem, L. Nilsson, B. Winblad*

*Department of Pharmacology, University of Uppsala, Box 591, S-751 24 Uppsala, Sweden.*

Muscarinic receptors in different brain regions and in peripheral blood cells have been studied in our laboratory using various receptor binding techniques and release studies.

In the human hippocampus a significant decrease with age (0–100 years) was found in the number of muscarinic receptors. In the thalamus on the other hand, an increased number of muscarinic binding sites was observed in the age range of 60–90 years. These age effects were not so obvious in Alzheimer's disease and were lost in multi-infarct dementia. Since studies in whole brain regions may overlook an internuclear regional variation we have measured the topochemical localization of muscarinic receptors in different brain regions. We obtained, for example, in the putamen of Alzheimer patients an increased number of binding sites in the whole antero-posterior direction. A method has been developed to dissect brain tissue with short post-mortem delay, freeze it in sucrose to −70°C and thereby keep the functional integrity. After preloading with the precursor [ $^3$ H]choline both a spontaneous and  $K^+$  induced release of [ $^3$ H]acetylcholine was obtained from cortical slices of control and Alzheimer patients. We think this may be a new promising technique for studying presynaptic receptor function in human brain tissue from individuals with different dementia disorders.

Peripheral blood cells such as lymphocytes have specific muscarinic binding sites. Recently obtained data in our laboratory indicate a decreased number of muscarinic receptors on lymphocytes from patients with Alzheimer's disease compared to age-matched controls. No significant change in number of binding sites was obtained on lymphocytes from patients with Parkinson's disease or multi-infarct dementia. This new approach offers the advantage that the patients can be followed prospectively and biochemical changes may be correlated to the severity of the symptoms of the disease.

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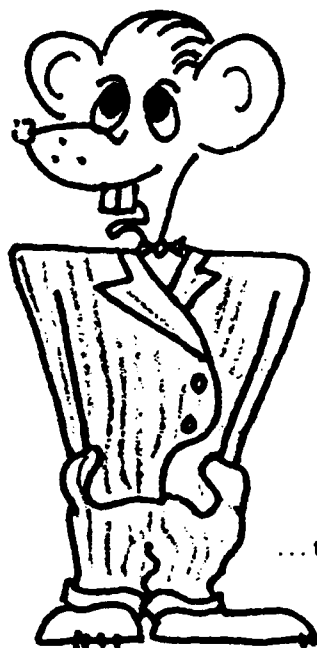
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